

**Binding Characteristics and Localization of *Arabidopsis thaliana* Ribosomal Protein S15a
Isoforms**

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ABSTRACT

Ribosomes which conduct protein synthesis in all living organisms are comprised of two subunits. The large 60S ribosomal subunit catalyzes peptidyl transferase reactions and includes the polypeptide exit tunnel, while the small (40S) ribosomal subunit recruits incoming messenger RNAs (mRNAs) and performs proofreading. The plant 80S cytoplasmic ribosome is composed of 4 ribosomal RNAs (rRNAs: 25-28S, 5.8S and 5S in the large subunit and 18S in the small subunit) and 81 ribosomal proteins (r-proteins: 48 in the large subunit, 33 in the small subunit). RPS15a, a putative small subunit primary binder, is encoded by a six member gene family (*RPS15aA-F*), where *RPS15aB* and *RPS15aE* are evolutionarily distinct and thought to be incorporated into mitochondrial ribosomes. *In vitro* synthesized cytoplasmic 18S rRNA, 18S rRNA loop fragments, and *RPS15a* mRNA molecules were combined in electrophoretic shift assays (EMSAs) to determine the RNA binding characteristics of RPS15aA/-D/-E/-F. RPS15aA/F, -D and -E bind to cytoplasmic 18S rRNA in the absence of cellular components. However, RPS15aE r-protein tested that binds mitochondrial 18S rRNA. In addition, RPS15aA/F only binds one of three 18S rRNA loop fragments of helix 23 whereas RPS15aD/-E bind all three 18S rRNA helix 23 loop fragments. Additionally, RPS15aD and RPS15aE did not bind their respective mRNA transcripts, likely indicating that this form of negative feedback is not a post-transcriptional control mechanism for this r-protein gene family. Furthermore, the addition of RPS15a transcripts to the EMSAs did not affect the binding of RPS15aA/F, -D and -E to 18S rRNA helix 23 loop 4-6, indicating that rRNA binding is specific. Supershift EMSAs further confirmed the specificity of RPS15aA/F and RPS15aE binding to loop fragment (4-6) of 18S rRNA. Taken together, these data support a role for RPS15a in early ribosome small subunit assembly.

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DEDICATION

This thesis is for the three strongest and most intelligent women in my life: my mother, my best friend, and my oma. Although my oma left me too soon, I will never forget the unconditional love and strength she brought to my life.

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LIST OF ABBREVIATIONS

A-site	aminoacyl site
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CDS	coding sequence
CLSM	confocal laser scanning microscopy
cryo-EM	cryo-electron microscopy
DEPC	diethyl pyrocarbonate
dsDNA	double stranded DNA
E-site	exit site
EF	elongation factor
EGFP	enhanced green fluorescent protein
EMSA	electrophoretic shift assay
ES	expansion segment
EST	expressed sequence tag
ETS	external transcribed spacer
IF	initiation factor
IGS	intergenic spacer
IPTG	isopropyl β -D-1-thiogalactopyranoside
IRES	internal ribosome entry site
ITS	internal transcribed spacer
LSM	laser scanning microscopy
LSU	large subunit
MS	mass spectrometry
mRFP	monomeric red fluorescent protein
mRNA	messenger RNA
Ni-NTA	nickel-nitrilotriacetic acid
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NOR	nucleolar organizing region
nt	nucleotide
OB	oligonucleotide binding
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pre-RNA	precursor RNA
P-protein	phosphorylated protein
P-site	peptidyl site
pI	isoelectric point
pol	polymerase
RACK	receptor of activated C-kinase
rDNA	ribosomal DNA
RNP	ribonucleoprotein
r-protein/RP	ribosomal protein

rRNA	ribosomal RNA
RT-PCR	reverse-transcription polymerase chain reaction
ssDNA	single stranded DNA
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
snoRNA	small nucleolar RNA
snoRNP	small nucleolar ribonucleoprotein
SSU	small subunit
TOP	terminal oligopyrimidine tract
tRNA	transfer RNA
UAS	upstream activation sequence
UTR	untranslated region

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Ribosomes synthesize proteins in all living cells via peptidyl transferase reactions. Both prokaryotic and eukaryotic ribosomes are membrane-free ribonucleoprotein (RNP) complexes composed of ribosomal proteins (r-proteins) and ribosomal RNAs (rRNAs). Eukaryotic ribosomes are greater in complexity and size than their prokaryotic counterparts as they contain longer RNAs and more r-proteins. The ribosome is made up of a large (50S-60S) and small subunit (30S-40S) which, upon assembly on messenger RNA (mRNA), becomes translationally active (Tate and Poole, 2004). The individual subunits have distinct biological functions: the large subunit catalyzes peptide bond formation between amino acids of a growing polypeptide chain, whereas the small subunit facilitates mRNA codon and transfer RNA (tRNA) anticodon interactions as well as mRNA proofreading functions. The translation of mRNAs is complex and the majority of research regarding ribosomes has focused on understanding this process. It is only recently that the three dimensional structure of the ribosome has been revealed, allowing for further investigation into its unique makeup. X-ray crystallography analysis and cryo-electron microscopy (cryo-EM) technology have allowed scientists to visualize the subunits at very low resolutions (Noller, 2001; Yusupov et al., 2001).

Ribosomes are found in all living organisms, and plants are unique in the types of ribosomes they contain (Warner, 2001). The plant cell contains eukaryotic 80S cytoplasmic ribosomes as well as prokaryotic-like 70S ribosomes in both mitochondria and plastids. Eukaryotic ribosomes are composed of four rRNAs, where the large subunit contains 25S, 5.8S, and 5S rRNAs and the small subunit contains an 18S rRNA. In Arabidopsis, the ribosome also contains 81 r-proteins (Barakat et al., 2001). The nucleolus, a sub-organelle composed of dense fibrillar, granular components and fibrillar centers, is the site of ribosomal subunit biosynthesis: the 25S, 18S, and 5.8S rRNAs are synthesized in the

nucleolus whereas in higher plants, the 5S rRNA genes are transcribed outside the nucleolus (Pederson and Politz, 2000). The majority of protein synthesis in all eukaryotes is facilitated by cytoplasmic ribosomes. In tobacco cells, the prokaryotic-like 70S chloroplast ribosome only synthesizes approximately 70 chloroplast proteins (Hirose and Sugiura, 2004).

Cytoplasmic r-proteins are synthesized in the cytoplasm and subsequently transported to the nucleus and accumulate in the nucleolus where ribosome subunit assembly occurs (Brown and Shaw, 1998). Proper assembly of ribosomal subunits is achieved by ensuring one copy of each rRNA and r-protein is incorporated into each ribosome (Chen and Huang, 2001). Following ribosomal subunit assembly, premature subunits are exported to the cytoplasm where subunit maturation occurs, and, depending on the requirements of the cell, the ribosomal subunits may assemble on mRNAs (Donovan and Pearson 1986; Fromont-Racine et al., 2003). As mentioned above, ribosomes generally contain only 1 copy of each r-protein (exceptions include the acidic phosphorylated proteins, P-proteins; in Arabidopsis there are four phosphoproteins, P0, P1, P2 and P3), however, the Arabidopsis genome contains ~254 r-protein genes, all of which can be grouped into multi gene families of between 2-7 (where at least 2 members of the same family are transcriptionally active) members that encode 81 r-proteins (Barakat et al., 2001). In contrast, the majority of r-protein genes within a gene family in mammals are pseudogenes (gene copies that do not produce a full-length functional protein) with only one functional, intron containing r-protein gene in each family (Kominami et al., 1981). In plants, some of the observed expression levels between different family members are spatially and/or temporally unique, which could indicate specialized functions for each family member, or perhaps more likely, unique modes of regulation of plant development where promoter elements have evolved responsiveness to different environmental triggers (Taylor et al., 1992). It has been suggested that at least 45% of r-proteins in plant 80S ribosomes are either different isoforms or post-translationally modified (Giavalisco et al., 2005).

r-proteins are relatively small (3.4-47 kDa) and basic, containing large proportions of lysine and arginine. r-protein functions include ensuring correct folding and stabilization of rRNAs, as well, some r-proteins may have extraribosomal functions such as aiding in DNA repair, binding translation factors, or important for signal recognition particle binding (Chavez-Rios et al., 2003; Halic et al., 2004). Although r-proteins are a major constituent of

ribosomal subunits and perform essential functions, surprisingly few have been well characterized.

The following thesis is an investigation into the RNA binding capabilities of the evolutionary divergent RPS15a isoforms in the model flowering plant, *Arabidopsis thaliana* (*A. thaliana*). The prokaryotic ortholog of RPS15a is the highly conserved 16S rRNA primary binding r-protein RPS8. In addition to its role in small subunit assembly, RPS8 regulates the expression of the *spc* operon via autogenous regulation. *Arabidopsis* RPS15a is encoded by a gene family comprised of six members, *RPS15aA-F* (Bakarat, 2001), resulting in 5 possible isoforms of the protein. The six *RPS15a* genes are distributed across all 5 *Arabidopsis* chromosomes although *RPS15aC* is not expressed (Hulm et al., 2005). Type I *RPS15aA/F* and *-D* encode proteins with 98-100% amino acid identity (*-A* and *-F* 100%) whereas the type II r-proteins RPS15aB and RPS15aE share 92% amino acid identity.

This research examined whether RPS15a isoforms are putative primary rRNA-binders and/or regulate their expression via binding to their respective mRNAs. Through the use of electrophoretic shift assays (EMSAs), the first binding studies involving RPS15a determined that both cytosolic RPS15aA/F and *-D* and mitochondrial RPS15aE can bind cytoplasmic 18S rRNA and specific 18S rRNA loop fragments of helix 23. It was also determined that type II RPS15aB and RPS15aE localize to mitochondria in tobacco leaf epidermal cells. Consistent with the localization pattern observed for RPS15aE, additional EMSAs confirmed that only it (no type I isoforms) interacted exclusively with mitochondrial 18S rRNA. These experiments support a role for type II r-protein RPS15aE as a functional homologue of RPS8 in mitochondria. A putative 18S rRNA binding site for RPS15a was also identified on helix 23 and allowed comparison of rRNA binding sites with other members of the S8p r-protein family.

1.2. Ribosome structure and functions

1.2.1 Ribosomal RNA

The deletion of a single nucleotide within 23S rRNA, G2252 (Samaha et al., 1995), along with the previous 23S rRNA mutants (Gourse et al., 1982; Skinner et al., 1985) demonstrated the importance of rRNA within the ribosome, although researchers would not be able to fathom the extent of rRNA's role in protein synthesis. Despite the overwhelming

early biochemical evidence (Barta et al., 1984; Noller et al., 1992) to support the hypothesis that large subunit rRNA is the catalytic entity of the ribosome, the majority of researchers would only consider large subunit rRNA as having a “possible involvement” (Nitta et al., 1998) in peptide bond formation. It was not until structural data of the ribosome first emerged to show that 23S rRNA is responsible for catalyzing the peptidyl transferase reaction (Ban et al., 2000). rRNA gained further scientific importance and recognition when the term “ribozyme” re-emerged (Cech, 2000). Based on crystal structures of the active center of the ribosome, it was determined that no protein is within 18 Å of the catalytic site (Ban et al., 2000). Some of the biological importance of RNA through X-ray crystallography analysis of RNA structure has been uncovered, although all of its functional capabilities have yet to be determined.

Nuclear magnetic resonance (NMR) and X-ray crystallography studies on rRNA structure have provided details on its makeup and general conformation (Ban et al., 2000; Schluenzen et al. 2000; Wimberly et al., 2000). rRNA at the structural level is more conserved, relative to ribosomal DNA (rDNA) sequence identity, between prokaryotes and eukaryotes. The small subunit rRNA (16S-18S) component illustrates this concept as its overall secondary structure is conserved among prokaryotic, plastidic, and eukaryotic small subunit RNAs. The small subunit rRNA has three major domains, central (also referred to as platform), 5' (also referred to as body), 3' major (also referred to as head) and one minor 3' domain, shown in Figure 1.1. Each domain of the rRNA is important to ribosome function. For example, in addition to being involved in ribosomal subunit association, the 3' domain interacts with incoming mRNA transcripts, initiation factor (IF) 2, elongation factor (EF) G, and several termination factors (Arkov et al., 1998; Wakao et al., 1991; Wilson and Noller, 1998). Crystal structure analysis of 16S rRNA discovered ~55 A-minor motifs, an abundant motif in rRNA, particularly important for long range helical interactions, and characterized by several adenines in minor grooves of neighboring helices that form hydrogen bonds with the 2' hydroxyl (OH) of G/C pairs in order to stabilize the helices (Wimberly et al., 2000). For example, there is a direct interaction between helix 13 and 44 in 16S rRNA, demonstrating that different domains of rRNA are connected to form its intricate 3-

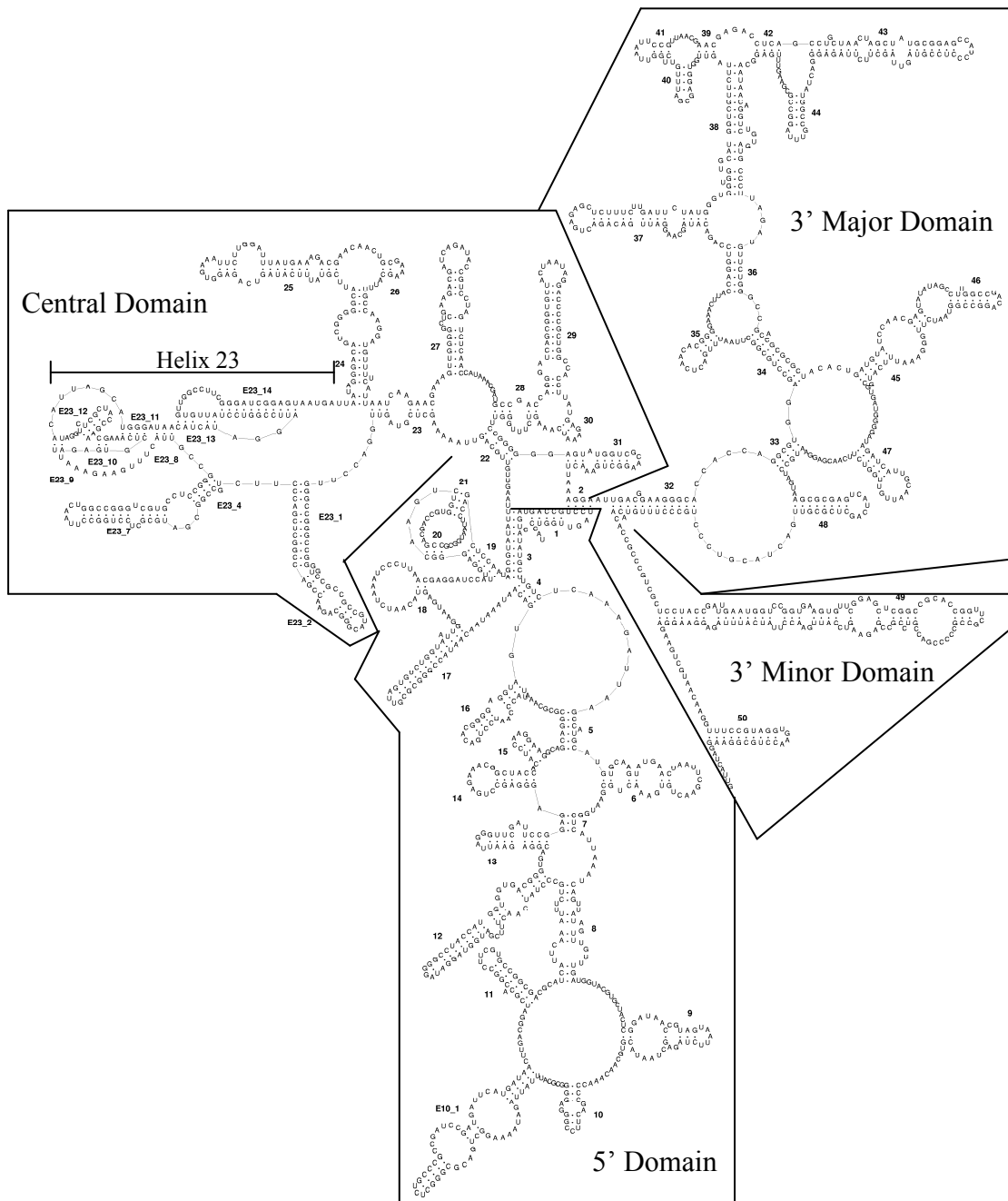


Figure 1.1. Predicted maize 18S rRNA secondary structure with domain organization indicated. This rRNA model and the helix numbers marked are in accordance with Wuyts et al. (2000). Figure adapted from van de Peer et al. (2000).

dimensional nature. Although the full significance of A-minor interactions is not yet clear, it has been suggested that they aid in conformational changes, such as molecular rearrangement during translocation (discussed in section 1.2.2.1).

The large ribosomal subunit (containing 23S-29S, 5.8S and 5S rRNAs) consists of six domains (I through VI) with much greater complexity (Noller, 2005). Potential contacts between domains have been identified via cross linking experiments: domains II and III, domains II and V, and domains IV and V. Not only do some of the domains come into close contact, but they are also functional. Domain VI contains a highly conserved sarchin/ricin loop, and its unique structure provides a basis for binding to EG-Tu and EG-F and is considered essential (Correll et al., 1998). In 23S rRNA, domain V harbors the peptidyl transferase center and also acts as a moderator of nascent protein folding (Chowdhury et al., 2002). Together, domains IV and V are considered essential for protein synthesis (Noller et al., 2000). Although the precise role of 5S rRNA is not fully understood, it has been suggested that it is involved in transmitting signals to the various ribosomal centers such as the decoding center, the peptidyl transferase center, the GTPase associated center, and to elongation factor binding elements (reviewed in Dinman, 2005).

By itself, it is difficult for RNA to form intricate structures due to its limited geometry. In the ribosome, short RNA helices contain Watson and Crick base pairs which can connect to longer or shorter loops (bulged or internal). These helices-loop connections can also contain non-canonical base pairs and aid in the formation of bends in the RNA. Hairpin loops, first determined by NMR, are important for proper RNA folding, binding regions for proteins, and exposing regions for base pairing with other RNA molecules (Varani et al., 1991). Other structures that are important for protein binding and recognition are bulges, and internal loops, which are divided into G/A mismatches, loop E family, loop B family, and sarchin loops (examples of some of the distinct loop types that are found in RNA, Shen et al., 1995; Noller, 1984). As well as the complex secondary and tertiary structures of rRNAs, their synthesis is tightly regulated. rRNA is encoded by highly conserved rDNA that is used extensively to characterize the evolutionary history of organisms through phylogenetic trees and predict secondary structures (Schnare et al., 1996; Soltis et al., 1999).

1.2.1.1. Ribosomal RNA genes

Ribosomal RNA gene sequences are conserved in all domains of life and have been used in phylogenetic studies for several decades. Prokaryotic rRNA genes, depending on the species, encoding the 23S, 16S, and 5S rRNAs, are present within the genome either in a single operon or in multiple operons spread throughout the genome. The Ribosomal RNA Operon Copy Number Database (<http://rrndb.cme.msu.edu/>) estimates that prokaryotes can have anywhere from 1 (e.g. *Thermoproteus tenax*) to 15 (e.g. *Clostridium paradoxum*) copies of any given rRNA operon (Klappenbach et al., 2001). The rRNA genes are linked in operons, most likely to ensure that each rRNA gene is transcribed in equimolar amounts. The rRNA genes are separated by internal transcribed spacers (ITSs), sequences of RNA that are removed from the precursor rRNA (pre-rRNA) during processing events. rRNA genes are also flanked by 5' and 3' external transcribed spacers (ETS) and several tRNA genes. For example, in *Agrobacterium tumefaciens*, the rRNA operon consists of: 16S rRNA gene-ITS [within this ITS are tRNA^{Ile} and tRNA^{Ala} genes]-23S rRNA gene-ITS-5S rRNA gene-tRNA^{Met} gene (Bautista-Zapanta et al., 2002). Between prokaryotes, the 16S rRNA gene contains both conserved and non conserved regions (Fisher et al., 2004). The chloroplast and mitochondrial rRNA gene operons generally contain prokaryotic-like promoters, with chloroplast promoters containing some signature motifs (such as positions -10 and -35 relative to the 16S rRNA sequence [Kössel et al., 1993]).

Nucleolar organizer regions (NORs) are chromosomal loci comprised of transcriptionally active or inactive rRNA gene arrays (McStay, 2006). Eukaryotic rDNA is found in multiple copies and often in repeats (Lafontaine and Tollervey, 2001). Like prokaryotes, the eukaryotic 35S rRNA precursor gene (18S-5.8S-23S), which is transcribed by RNA polymerase (pol) I, also contains non-transcribed intergenic spacer (IGS) segments within the operon unit (Zomerdijsk and Tjian, 1998). Vertebrates contain a long precursor rRNA gene arrangement of: 5'ETS-18S-ITS1-5.8S-ITS2-28S-3'ETS, where in this arrangement, a spacer promoter (found within the intergenic region of rRNA gene clusters and influence transcription of pre-rRNA), enhancer region, and the complete gene promoter are directly upstream of the 5' ETS (Caburet et al., 2005). With the exception of *Saccharomyces cerevisiae* (*S. cerevisiae*), the 5S rRNA gene is located outside the NORs and its copy number varies greatly between species (Neigebom and Warner, 1990; Oakes et al.,

2006). *S. cerevisiae* rDNA repeats (each repeat being ~ 9.1 kb) are located within one NOR (chromosome 7), and found in ~150 tandemly repeated copies (Kim et al., 2006). Similarly, in humans, the rRNA gene arrays (precursor of 28S, 18S, and 5.8S) have been mapped to NORs on chromosomes 13, 14, 15, 21, and 22 (Kenmochi et al., 1998; Worton et al., 1988), and contain approximately 160-200 repeats, while 5S rRNA gene repeats are found on chromosome 1 (Dechampsme et al., 1999). Between eukaryotes, there appears to be rDNA heterogeneity, mostly due to differential lengths of the spacer region sequences (Gonzalez, et al., 1985). In mice, the repeat unit is approximately 44 kb (Kominami et al., 1981) and there appears to be repetitive sequences in the non-transcribed spacer regions (i.e. IGSs), that are not necessarily found in the organization of other species' rRNA gene and spacer regions (e.g. *Xenopus laevis*)

The organization of rDNA promoters is similar in all eukaryotic rDNA genes. Although there is a low level of sequence identity among most eukaryotic promoters for RNA pol II transcribed genes (Marilley and Pasero, 1996), the promoters of rDNA contain two distinct domains: the first domain, referred to as the “core promoter”, is ~45 base pair (bp) long and contains the transcription start site, while the second domain is located at position -150 bp (relative to the start site of transcription) and appears to function in enhancing transcription (Henderson and Sollner-Webb, 1990). Several structural features (i.e. specific curvatures, bending elements involved in the shape of the promoter region, and twist angles) of these promoters are conserved in eukaryotes (Marilley and Pasero, 1996). Plant rRNA genes vary in both copy number and IGS length (Rogers and Bendich, 1987). The haploid *A. thaliana* genome contains 570 rRNA genes (spread over two NORs located on chromosomes 2 and 4), several of which are surrounded by gene spacer sequences (Doelling et al., 1993; Pontes et al., 2003). This low number is in contrast to other plants, such as certain varieties of flax, which have more than 1200 rRNA gene repeats (in the haploid genome). Plant rRNA gene promoters are highly conserved across the kingdom. The Arabidopsis rDNA gene promoter consists of a TATA box and four to six guanosines (located upstream of the core promoter site) and it has been hypothesized that plant rDNA promoters are similar to those of protein-coding genes (Doelling and Pikkard, 1995). It is clear that over the course of evolution, eukaryotes have acquired many more rRNA gene copies compared to prokaryotes, although the organization of these genes has been retained.

1.2.1.2. The processing of ribosomal RNA

Several types of RNase molecules are responsible for post-transcriptional modifications and processing of pre-rRNA transcripts in both prokaryotes and eukaryotes. The prokaryotic rRNA operon(s) is transcribed as a 30S precursor molecule that is processed by several endo- and exonucleases. Exoribonucleases RNase T and RNase E both process pre-rRNA, while RNase T also processes tRNA and the 3' terminus of 23S rRNA (Li et al., 1999). Often, 23S and 16S rRNA genes are flanked by complementary base pairs that interact to form stem structures which RNase III cleaves (Allas et al., 2003), and which are subsequently subjected to further processing by other RNase complexes, releasing the individual pre-rRNA molecules (Matsunaga et al., 1996, Sun et al., 2004). Further processing of pre-rRNA, such as structural changes to 16S rRNA, occurs during ribosome subunit assembly.

The nucleolus is the site of rDNA transcription and processing and in eukaryotes, RNA pol I is responsible for synthesizing the pre-rRNA units. In higher eukaryotes, the rRNA processing scheme is comparable to that established for yeast. The small-subunit (SSU) processome is a large nucleolar RNP composed of at least 40 proteins and U3 small nucleolar RNA (snoRNA) that is required for processing of the SSU rRNA (Bernstein et al., 2004). In yeast, the 35S pre-rRNA transcript is cleaved by the SSU processome at the 5' ETS generating a 33S pre-rRNA. Further cleavage of the 33S pre-rRNA specifically, at the 5' end of 18S rRNA (also facilitated by the SSU processome), generates the 32S pre-rRNA. The final cleavages at the internal ITS1 of 32S pre-rRNA generate the 20S and 27S pre-rRNAs. In order to obtain final fully processed rRNA molecules, the 20S rRNA (and associated r-proteins) is exported into the cytoplasm to become fully functional 18S rRNA (in yeast; Vanrobays et al., 2003). It was initially believed in mammals and plants that this cleavage occurred in the nucleus, however, recent evidence demonstrated that in mammals the final cleavage at the 3' end of the 18S rRNA and the small subunit maturation steps occur in the cytoplasm (Rouquette et al., 2005). 27S rRNA is further processed in the nucleolus by RNase MRP to produce functional 23S rRNA and 5.8S rRNA. In plants, it is understood that all of the pre-rRNA processing steps occur exclusively in the various components of plant nucleoli (Brown and Shaw, 1998). The majority of pre-rRNA processing is facilitated by non-ribosomal proteins and small nucleolar ribonucleoprotein complexes (snoRNPs), as well

as the eukaryotic ortholog to bacterial RNase III (found in all eukaryotes, Filippov et al., 2000).

As well as the processing steps, pre-rRNA is also subjected to base modifications prior to correct folding and final stabilization of the 18S, 25S, and 5.8S rRNAs. In eukaryotes, specific types of modifications are required during rRNA processing: 2'-O-methylations (to ribose sugars), methylations to bases (Kiss-Laszlo et al., 1996), and the addition of pseudouridines to modify specific regions (determined by snoRNA, which are also implicated in pre-rRNA folding) of the 18S, 25S and 5.8S rRNA precursors (20S and 27S pre-rRNAs, respectively [Eichler and Craig, 1994; Ganot et al., 1997]). In humans, these modifications occur at 100 rRNA sites, whereas in yeast, only 50 rRNA sites are modified. In *E. coli*, this number decreases to only 14. In most eukaryotes, binding of ribosomal proteins (and non ribosomal proteins in yeast) to pre-rRNA occurs prior to cleavage. Subsequent cleavage of the ITS and ETS regions, which can occur through a number of different pathways, results in mature 18S, 25S, and 5.8S rRNAs (Michot and Bachellerie, 1991). It is interesting that the small subunit pre-35S rRNA associates early with the proteins required for its maturation into the 40S subunit, whereas the maturation machinery required for 60S large subunit formation binds much later (Schäfer et al., 2003).

rRNA processing in plants is very similar to that described above, although details regarding processing sites is lacking. Plants contain specific snoRNA gene families including the U3 family (transcribed by RNA pol III), and the U14 family which are transcribed from small nuclear RNA promoter elements (reviewed in Brown and Shaw, 1998). Unlike vertebrates, plant snoRNA genes are expressed as polycistrons (Leader et al., 1997), and some snoRNA genes are located within pre-mRNA introns (Barneche et al. 2000). Arabidopsis rRNA undergoes extensive modification; ~ 120 nucleotides are methylated while > 100 uridines are converted to pseudouridines. These modifications are mainly found at functional rRNA domain sites, such as in the peptidyl-transferase centre (Maden, 1990). Correct processing of pre-rRNA is a key to ribosome biogenesis.

1.2.1.3. Ribosomal subunit structure

The first general structural observations of the ribosome in the early 1970s identified preliminary locations for most ribosomal components. Early electron microscopy and later

cryo-EM analysis provided the first low resolution, and subsequently higher resolution, views of ribosomal subunits and the complete ribosome (Shen et al., 1995). Later, X-ray crystallography produced atomic resolution (4.5 Å) structures of the prokaryotic ribosome, particularly the 50S *Haloarcula marismortui* (*H. marismortui*) subunit (Ban et al., 2000) and the 30S *Thermus thermophilus* (*T. thermophilus*) subunit (Wimberly et al., 2000). These studies provided details of overall architecture of rRNA interactions with r-proteins, rRNA folding, and rRNA stem loops as well as providing some indication of how tRNAs interact with the ribosome (Brodersen et al., 2002; Schuwirth et al., 2005; Yusupov et al., 2001). Further crystallography studies on the prokaryotic ribosome have provided a structural basis for the unique functions of the ribosome.

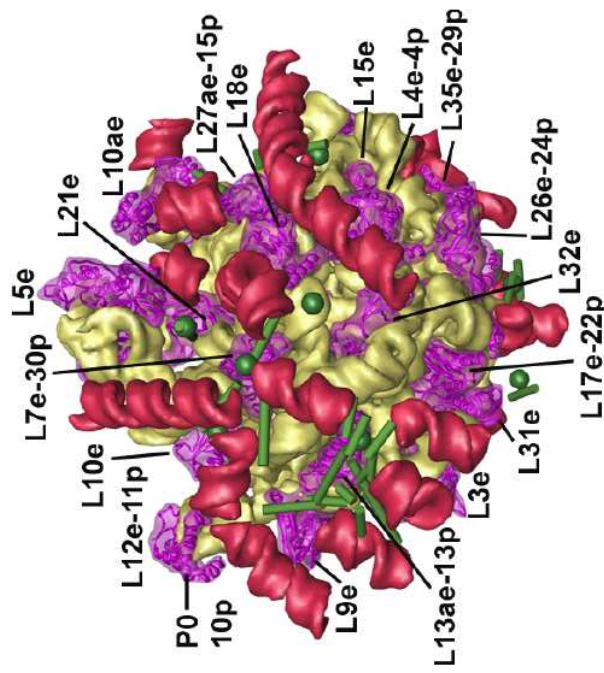
The prokaryotic 70S ribosome has a molecular mass of ~ 2.4 MDa. In prokaryotes, the primary function of the small 30S subunit is the decoding of genetic information. Six structural domains, the body (RPS4, RPS5, RPS8, and RPS16), neck, head (RPS2, RPS3, RPS9, RPS10, and RPS14), platform (RPS6, RPS11 and RPS18), spur and helix 44 regions, have been identified in the small subunit (William et al., 1999). The large 50S subunit contains both the 23S and 5S rRNAs and has a diameter of 250 Å. 23S rRNA also has six structural domains that are distinct from one another in shape and organization (Klein et al., 2004). In addition, cryo-EM images of the large subunit have highlighted three finger-like projections, the L1 stalk, central protuberance, and the L7/L12 stalk (Marquis et al., 1981). The mushroom shaped domain I is located behind the L1 projection; the largest domain, domain II, comprises three protrusions, one being the L7/L12 stalk; domain III is compact with four stem loop pointed regions giving it a star like appearance; domain IV is important in 30S ribosomal subunit contact and is unique in that it does not depend on r-proteins for stability to the same degree as the other domains; domain V contains the peptidyl transferase activity (discussed in section 1.2.2.1); the smallest domain, domain VI, consists of stem loop 95, which is essential for protein factor binding during ribosomal subunit assembly (Ban et al., 2000; Lescoute and Westhof, 2006). X-ray crystallography resolutions (2.4 Å) of the large subunit have identified the peptide tunnel structure that runs through the large subunit, a channel that had previously been hypothesized to facilitate the movement of polypeptides as they are being synthesized. It was confirmed that the tunnel, extending from the middle of the subunit to the bottom backside, exists and that specific proteins are associated with its

shape: RPL4 and RPL22 form a bend in the tunnel. The entrance of the peptidyl exit tunnel is surrounded by a domain V loop of 23S rRNA (where 15 proteins interact with the domain), A and P loops, and extension r-proteins RPL2, RPL3, RPL4, and RPL10, which are embedded into the peptidyl transferase active site of the tunnel opening and may stabilize this structure (Nissen et al., 2000).

Structural analysis of the 80S eukaryotic ribosome comes from cryo-EM studies (~ 8 Å; Spahn et al., 2001). The cryo-EM analyses of 80S ribosomes from yeast (Spahn et al., 2001), mammals: rabbit (Morgan et al., 2000), canine (Chandramouli et al., 2008), see Figure 1.2; human (Spahn et al., 2004), fungi (Nilsson et al., 2007), and wheat germ (Halic et al., 2004), indicate the general architecture of eukaryotic ribosomes is similar, with differences in overall size largely due to variation in the number of expansion segments (ES), hypervariable inserts responsible for altered morphology of eukaryotic ribosomes relative to ribosomes of prokaryotes (Hancock and Dover, 1988). Particularly, the expansion segments ES27, essential for yeast viability (Sweeney et al., 1994), and ES7/ES39 of 25S rRNA in yeast are different to all other 80S ribosomes studied. In fungi, ES27 is largely absent and the position of ES7/ES39 adopts a different conformation to that found in any other eukaryotic ribosome. Other slight differences between eukaryotic ribosomes include changes in the P-stalk region, although it appears that the main differences lie in expansion segment number, length, and position which could reflect a requirement to regulate factor binding.

The eukaryotic small subunit (40S) of *S. cerevisiae* contains a larger rRNA molecule (18S) than the *Escherichia coli* (*E. coli*) 30S ribosomal subunit (16S) containing some helix position changes, 11 more r-proteins, and some variation in overall shape due to these differences. The 40S subunit is similar to its 30S prokaryotic counterpart in that it is comprised of a head, body, platform, and helix 44. In eukaryotes, these same structures are made from 18S rRNA and 32 r-proteins along with three additional expansion segments, ES12, ES6, ES3 (Alkemar and Nygård, 2006). The eukaryotic large ribosomal subunit contains an additional rRNA molecule, 5.8S rRNA, longer rRNA molecules, and more proteins with respect to its eubacterial and archaebacterial counterparts (Ali et al., 1999).

A)



B)

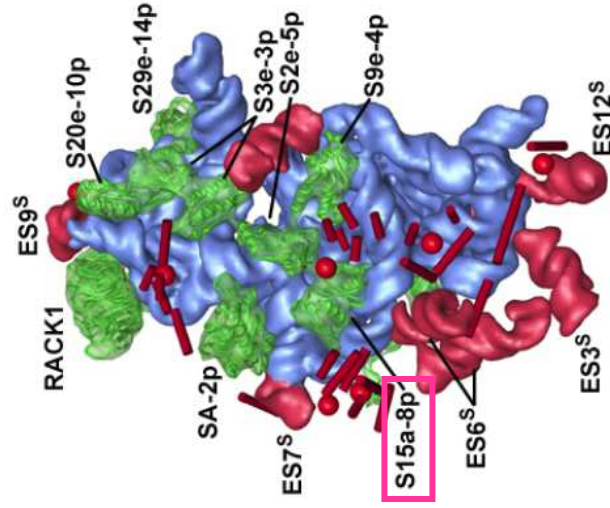


Figure 1.2. Modeled proteins in the large (60S) and small (40S) ribosomal subunits of the canine cytoplasmic 80S ribosome. A) A back view of the 60S subunit highlighting the distribution of modeled large subunit r-proteins superimposed on 25S rRNA. B) A back view of the 40S subunit highlighting conserved small subunit r-proteins superimposed on 18S rRNA with expansion segments shown on both subunits. RPS15a is boxed in pink in the small subunit. Figure adapted from Chandramouli et al., 2008.

The overall structure, however, of the 60S large subunit is quite similar to the *E. coli* 50S large subunit with the exception of some changes in helix positions: helices 43, 44, and 78 positions are quite different in eukaryotes relative to the *H. marismortui* subunit (Nissen et al., 2000). In addition, similar to 18S rRNA, there are expansion segments present in the large subunit 5.8S and 25S rRNAs, along with four additional bridges (apart from the seven already present in the prokaryotic 23S rRNA) that link to the 40S subunit (Spahn et al., 2001). The intersubunit bridges likely play a role in ratchet-like subunit rearrangement and there are specific bridges only identified in eukaryotic ribosomes that have been implicated in resetting the ribosome's conformation during ratchet-like rearrangement (Chandramouli et al., 2008). It will be interesting to further compare conserved and non-conserved regions of eukaryotic ribosomes when X-ray crystal structures become available.

1.2.2. Eukaryotic ribosome biogenesis

Ribosomes are produced based on the growth requirements of cells, regulating the rate of rDNA transcription, with ribosome biogenesis responding accordingly (Savino et al., 2001). Eukaryotic ribosome subunit biogenesis occurs in the nucleolus, a substructure within the nucleus composed of rDNA (transcribed during the S and G2 phases of the cell cycle) and proteins surrounded by nucleoplasm (Chen and Huang, 2001). Nucleolar components involved in ribosome biogenesis shuttle between the nucleolus and nucleoplasm during interphase of the cell cycle. The biogenesis of eukaryotic ribosomes is a multi-step process (Venema and Tollervey, 1999) with maturation of rRNA, from pre-rRNA, occurring in the nucleolus concurrently with ribosome subunit assembly (Fromont-Racine et al., 2003). Extranucleolar transcription of 5S rDNA is carried out by RNA pol III and is followed by import of the 5S rRNA into the nucleolus for ribosome subunit assembly (Willis, 1993). A large number of proteins are involved in nucleolar ribosome subunit assembly; in mammals, it is predicted that ~200 accessory proteins and numerous snoRNPs are involved (Fromont-Racine et al., 2003).

R-protein genes are transcribed in the nucleus by RNA pol II, and the processed r-protein mRNA is exported to the cytoplasm where it is translated to produce r-proteins (Brodersen and Nissen, 2005). The majority of newly synthesized r-proteins are then imported into the nucleus: RPL23a can interact with at least four distinct import proteins

(importin, transportin, importin 5 and importin 7) via its beta-like import receptor binding domain (Jäkel and Görlich, 1998). In the nucleolus, r-proteins assemble with rRNA molecules to form the pre-small and pre-large ribosomal subunits. Assembled pre-subunits are exported from the nucleus to the cytoplasm via export pathways that appear to be conserved in both animals and plants: in yeast, the nuclear export of the 60S ribosomal subunit is facilitated by chromosome region maintenance protein 1 (Crm1), RanGTP and the adaptor protein Nmd3 (Ho and Johnson, 1999) while the 40S subunit export is facilitated by the same export receptor, with a different as yet unidentified adaptor protein (Johnson et al., 2002). While the specific ribosomal subunit export receptors of plants are unknown, a similar pathway could be in operation as Arabidopsis orthologs to Crm1 (Atxpo1) and nmd3 have been identified (Haasen et al., 1999). In the cytoplasm, mature ribosomal subunits are formed with the binding of final r-proteins, while non-ribosomal proteins such as Dim1p, a methyltransferase, and Tsr1p are required for the final cleavage of 20S rRNA to 18S rRNA, to form the complete mature small subunit (Schäfer et al., 2003). This final step allows the small ribosomal subunit to assemble on mRNA, facilitating large subunit association to form the complete ribosome (Semrad et al., 2004).

1.2.2.1. Ribosome function

Protein biosynthesis is catalyzed by the ribosome in all living organisms. It is understood that ribosomal subunits prior to initiation of translation exist as free units until assembly of the 30S subunit with initiation factors, an mRNA transcript, and an initiator tRNA^{Met} (Noller, 2005). Translation in eukaryotes is fairly similar to that in prokaryotes with some exceptions (Merick, 2003). The initiator methionine is not the modified formylmethionine of prokaryotes and different IFs, EFs, as well as a cap-binding protein are involved in initiation and elongation (Arndt and Kane, 2003). The 60S subunit binds to this initiation complex to form the 80S functional ribosome. The detailed structure of the two subunits has allowed for insight into domain organization of subunits, structural components of the subunits, and rRNA-protein interactions (Jenni and Ban, 2003). In prokaryotes, binding of the 50S subunit to the 30S initiation complex releases initiation factors and the resulting 70S ribosomal complex now contains three binding sites, aminoacyl (A) for aminoacyl tRNAs, peptidyl (P), and exit (E) (Selmer et al., 2006). These tRNA binding sites

on the ribosomal complex facilitate initiation, elongation and termination, respectively. Polypeptide synthesis and the steps that lead up to it are highly organized (Agmon et al., 2003).

More recently, the ribosomal subunits have been depicted as regulatory elements, selecting specific mRNAs and directly effecting translation efficiency. It is thought that both r-proteins and rRNAs interact at specific sites on select mRNAs. The ribosome filter hypothesis (Mauro and Edelman, 2002) postulates that various mRNAs compete for ribosome binding, and this competition is controlled by ribosome heterogeneity. Some mRNA molecules contain sequences complementary to rRNA, particularly small subunit rRNA, leading to speculation that r-proteins and rRNA would be able to either bind or base pair to the complementary sequences in mRNA in order to effect regulation of translation. Specifically, the ribosome filter hypothesis suggests that mRNA binding sites might act as an internal ribosome entry site (IRES), a sequence within the 5' untranslated regions (UTRs) characterized by their extensive tertiary structure, which binds the 40S subunit to initiate translation. These sequences are well defined in viral systems (Spahn et al., 2001b; Tsukiyama-Kohara et al., 1992; Wang et al., 1993). It has been shown that during times of stress, when cap dependent translation is often repressed, mRNA molecules with IRES sequences are in abundance (Lang et al., 2002). In addition, the Arabidopsis RPS18C mRNA, which contains a 15 nt sequence with 100% complementarity to the 3' end of the 18S rRNA, was not only most abundantly transcribed, but the sequence mediated cap-independent translation (Vanderhaeghen et al., 2006). Some viral IRESs can recruit the 40S ribosomal subunit independently of any initiation factors (Wilson et al., 2000). As well as the mRNA sequences, the hypothesis identifies regions on mRNA molecules that the 40S subunit may potentially interact with: the 3' poly(A) tail, the 5' 7-methylguanosine cap, or loop structures with the aid of specific initiation factors. Although the ribosome filter hypothesis suggests enhanced translation, it was shown that too much nucleic acid identity between the mRNA sequences and 18S rRNA results in poor translation (Verrier and Jean-Jean, 2000). These results suggest that the degree of complementarity between mRNA and rRNA might regulate translation efficiency.

Although the effects of ribosomal protein heterogeneity on translational efficiency is poorly understood, a potential role of P2 heterogeneity in P-protein composition, in relation

to regulation of translation in maize, was discussed when developmentally and environmentally regulated heterogeneity was observed in their acidic r-protein levels and phosphorylation states (Szick-Miranda and Bailey-Serres, 2000). Again the functional significance of these findings is unknown. Szick-Miranda and Bailey-Serres (2000) used a model to illustrate different types of ribosomes resulting from P-protein heterogeneity and their putative functional significance. They postulated that variations in P-protein composition could influence elongation rates as well, and that differences in P-protein phosphorylation could have potential implications on translation as well as additional ribosomal heterogeneity. Further research surrounding the effects of ribosome heterogeneity (whether r-proteins or rRNA attribute to heterogeneity) on translational efficiency is required.

1.3. Ribosomal proteins (r-proteins)

R-proteins are major constituents of ribosomal subunits and can be responsible for up to one half of the total mass of a ribosome. Eukaryotic r-proteins are classified and claded into group I, group II or group III (as per Barakat et al., 2001) based on their homology to archaeobacterial and eubacterial r-proteins. Several prokaryotic and eukaryotic r-proteins have been isolated, although few have been characterized. The majority of proteomic characterization of large complexes has been facilitated by mass spectrometry (MS) which has been used to identify the majority of r-proteins to date, as well as specific post-transcriptional modifications. Generally, there is one copy of each r-protein per ribosome (exceptions are the acidic P-proteins), however, the total number of r-proteins per ribosome differs depending on the species; *E. coli* ribosomes contain 56 r-proteins (22 in the small subunit, 34 in the large subunit; Wittmann-Liebold et al., 1990), while both yeast and rat ribosomes contain 79 r-proteins (32 in the small subunit, 47 in the large subunit, Spahn et al., 2001; Wool et al., 1995). To identify the r-protein components of plant ribosomes, in the absence of crystal structures or other biochemical analyses, 2D gel electrophoresis followed by mass spectrometry analysis has been employed for wheat, soybean, barley, Arabidopsis, maize and tobacco. These analyses show that the Arabidopsis cytosolic ribosome contains 81 r-proteins (33 in the small subunit, 48 in the large subunit; Carroll et al., 2007, Chang et al., 2005, Yusupov et al., 2001). One of these proteins, P3, is a plant specific r-protein of the

60S ribosomal subunit. Unfortunately MS analyses cannot detect all of the r-proteins present for a given plant species, due to the limited tandem MS analysis of 1D SDS-PAGE separated r-protein bands that is generally performed, and routinely therefore underestimates the actual number of r-proteins present in a ribosome. For this reason, X-ray crystallography data is preferred although it is often difficult to obtain.

It was previously presumed that the functions of r-proteins were to stabilize and to ensure compact and correct folding of rRNAs in the ribosome (Klein et al., 2004). However, recent research suggests otherwise, with the possibility that some r-proteins have specific intraribosomal and extraribosomal functions such as signal recognition (Chavez-Rios et al., 2003) and halting elongation (Halic et al., 2004) which will be discussed below (section 1.3.2).

1.3.1. R-protein structure and biochemistry

R-proteins range in size depending on the species. *E. coli* r-proteins range from: ~4.3 kDa (RPL36) to ~61.1 kDa (RPS1) (Arnold and Rielly, 1999) while yeast r-proteins range from ~3.3 kDa (RPL41) to ~43 kDa (RPL3) (Lee et al., 2001). Arabidopsis is similar to yeast in that the r-proteins range in size from ~3.4 kDa (RPL41) to ~44.7 kDa (RPL4) (Barakat et al., 2001). In general, r-proteins contain a large proportion of basic amino acids (lysine and arginine) relative to acidic amino acids (aspartic and glutamic acid) although all eukaryotic ribosomes have an acidic r-protein stalk equivalent to the prokaryotic L7/L12 stalk (Mitsui et al., 1989). A large number of r-proteins found in the large subunit contain surface globular domains, thought to function in binding and stabilizing rRNA (Schuwirth et al., 2005). It is interesting that initiator or primary binding r-proteins in prokaryotes (RPS4, RPS7, RPS8, RPS15, RPS17 and RPS20 in the 30S subunit) contain a globular domain, further implicating this type of domain in putative rRNA stabilization (Brodersen et al., 2002). Another structural feature of many r-proteins is the presence of an extended basic tail that might function in holding rRNA structures together. Several primary binding r-proteins in the large 50S subunit contain this extended tail.

NMR spectroscopy is a tool to investigate r-protein topology and provides specific domain details. *E. coli* RPL25 contains a distinct β -barrel and α -helices (Stoldt et al., 1998), *Pyrococcus horikoshii* RPS28 contains a Greek key motif (common structural motif of four

β -strands) and several β -strands which form a structure similar to an oligonucleotide binding (OB) fold (five-stranded β -barrel arranged). Several r-proteins (including RPS1, RPS12, RPS17, RPL2) contain OB folds which have putative RNA binding capacities (Brodersen and Nissen, 2005; Theobald et al., 2003).

MS investigation of r-proteins has also proven useful to analyze post-translational modifications that potentially affect both structure and function of r-proteins. Post-transcriptional modifications include methionine removal, methylation, acetylation, and phosphorylation. Eukaryotic post-translational modification of r-proteins appears to be conserved: methylation of RPL1 (RPL10a in Arabidopsis), RPL12, and RPL42 (RPL33a in Arabidopsis) r-proteins in yeast and Arabidopsis has been reported. Conservation of specific r-protein modifications in eukaryotes suggests that they play an important role in function for a given r-protein. In the plant ribosomes so far analyzed, there appears to be a high level of post-translational processing of r-proteins. In eukaryotes (such as *Zea mays* [Z. mays], Williams et al., 2003; *Drosophila melanogaster* [D. melanogaster], Radimerski et al., 2000; and *S. cerevisiae*, Johnson and Warner, 1987), the well characterized RPS6 is rapidly phosphorylated at its C-terminus (Stewart and Thomas, 1994). Similarly in plants, RPS6 isoforms are phosphorylated and multiple phosphorylation sites at the C-terminus have been characterized (Barth-Baus et al., 2002; Fumagalli and Thomas, 2000).

The majority of structural research has focused on prokaryotic rRNA subunits and their r-proteins, with little exploration of eukaryotic or plant r-proteins. Additionally, little biochemical analysis has been performed on r-proteins. Recently, Giavalisco et al. (2005) showed a high level of heterogeneity within the 80S r-proteins of Arabidopsis: 45% of the r-proteins were either different isoforms or post-translationally modified. In Arabidopsis, 34% of r-protein families have two or more expressed members. Further characterization of plant r-proteins will shed light on the requirement for and function of plant 80S ribosome heterogeneity.

1.3.2. R-protein functions

Several questions surround the underlying mechanisms of ribosomal-tRNA-binding, translocation, and the peptidyl transferase center. Is rRNA solely responsible for performing these functions, or are r-proteins functionally relevant? The characterization of r-protein

mutants in bacteria and yeast has ascribed significant functional importance to r-proteins. Furthermore, mutant analysis in prokaryotes has demonstrated that the small subunit r-proteins RPS4 and RPS5 stabilize the interface between the shoulder and platform domains (Lodmell and Dahlberg, 1997). Atomic structural data for both prokaryotic large and small subunits has identified several r-proteins, with specific putative functions, located extremely close to their putative sites of action. tRNA decoding and recognition functions are mainly catalyzed by the RNA component of the ribosome, although r-proteins specific for these functional regions have been identified. RPS12 is important for tRNA decoding in the ribosomal A-site and is located in close proximity to the tRNA binding sites. Specifically, a region of RPS12 is physically involved in recognizing the correct tRNA (Bohman et al., 1984). Also, *E. coli* r-protein RPS7 interacts with E-site tRNA (of the three sites, E, P, A, the E-site contains the most r-proteins) in order to dislodge it from the ribosome. In *E. coli*, large subunit r-protein *rpl27* mutants are impaired in facilitating the correct movement of A-site bound tRNA to the peptidyl transferase center (Wower et al., 1998). For many r-protein mutants, the rate of protein synthesis either increases or decreases. However, for *E. coli rpl1* mutants, the rate of synthesis of RPL11 was greatly enhanced, highlighting an important function of several r-proteins in transcriptional and or translational regulation (Jinks-Robertson and Nomura, 1982). Structural analysis coupled with mutant characterization in *E. coli* has determined that r-proteins function in DNA repair, RNA processing, transcription, and translation (Wool, 1996).

R-protein mutational analysis has also proven a useful tool to dissect yeast r-protein functions. As described for prokaryotes, several yeast r-protein mutants also affect the kinetics of protein synthesis and translational accuracy. Yeast cells carrying *rpl24* deletions showed an increase in resistance to the translocation inhibitor cyclohexamide, indicating that r-protein may affect cyclohexamide binding. Indeed, X-ray crystal analysis of this arrangement confirmed this hypothesis. In addition, a lack of translational accuracy is reported for the yeast *rpl39* mutant (Dresios et al., 2000). In both prokaryotes and eukaryotes, small subunit RNA appears to be largely responsible for mRNA recognition, although *rps1* mutants in prokaryotes suggest that this protein plays a role in mRNA binding (Aliprandi et al., 2008). In eukaryotes, receptor of activated C-kinase (RACK) 1 plays a role in mRNA binding and activation of protein synthesis (Nilsson et al., 2004).

Not all r-proteins function primarily as rRNA binders or mRNA stabilizers. Additional functions for r-proteins combined with the discovery that decoding interactions are carried out exclusively by rRNA, suggests that r-proteins might have originally had specialized functions within and outside of the ribosome. Unfortunately, mutational analysis of r-proteins in the absence of available crystal structures can only highlight some of the direct or indirect functions of a particular r-protein. It will be crystal structures that provide details of r-protein-rRNA interactions and/or changes to the rRNA structure. Although there are several Arabidopsis r-protein mutants characterized, the absence of plant 80S ribosome crystal structures prohibits the analysis of the mechanism underlying r-protein functions. Impaired photosynthesis during Arabidopsis seedling development was observed for plastid *rps21* knockout plants (Morita-Yamamuro et al., 2004), whereas a partial suppression of *RPS6* in Arabidopsis results in abnormal organ formation (both flower and shoots), although other researchers suggest a more complex role for the RPS6 family (Morimoto et al., 2002). It has become apparent that phosphorylation of RPS6 causes selective translation of 5'-terminal oligopyrimidine tract (5' TOP) mRNAs (usually encode proteins involved in protein synthesis), although the mechanism underlying the selection remains a mystery (Terada et al., 1994). RPS6 phosphorylation-independent pathways for 5'TOP mRNA translation have also been uncovered (Barth-Baus et al., 2002) however, the proposed independent pathways cannot exclude the possibility that RPS6 phosphorylation has a role in regulating translation of 5'TOP mRNA. When atomic structures of the plant 80S ribosomes become available, positional information and structures of the plant r-proteins will aid in elucidating their functions.

1.3.3. R-protein genes

R-protein genes in many prokaryotes are arranged in operons (Mager, 1988). Eukaryotic r-protein genes appear to be dispersed non-randomly throughout the genome. Mapping r-protein genes in several organisms has proved difficult based on the presence of processed pseudogenes (Draper and Reynaldo, 1999). In several plant species, r-protein genes are present in gene families containing several members (in Arabidopsis 2-7) with the majority of the members being transcriptionally active (reviewed in McIntosh and Bonham-Smith, 2006). The majority of the 249 putative r-protein genes found in the Arabidopsis

genome appear to be expressed (Barakat et al., 2001). However, in mammalian genomes such as rat, there is an average of 12 members per gene family with only one actively transcribed member (Wool et al., 1996). In general, eukaryotic r-protein genes are highly conserved in terms of nucleotide sequence and intron position. Characterization of 73 human r-protein genes found them to contain approximately 5.6 exons per gene (Yoshihama et al., 2002), with GC rich promoters (some with TATA boxes), and fairly small 5' and 3' UTRs (sizes ranging from 56 bp to 4.4 kbp) which is comparable to mice r-protein genes (Hariharan and Perry, 1990). Due to reshuffling and extensive genome duplication in Arabidopsis (Blanc et al., 2000) it is difficult to analyze and compare ~249 r-protein gene sequences (Barakat et al., 2001), and therefore researchers tend to analyze r-protein gene families exclusively. Information on plant r-protein genes is limited, however it was determined that 81% of Arabidopsis r-protein genes contain telo-boxes (AAACCCTAA, in root primordial, these motifs are important for activating of gene expression, Tremousaygue et al., 1999), within their 5' regions (Tremousaygue et al., 2003). R-protein gene expression analysis also suggests that r-protein gene promoters contain elements that are likely responsive to developmental (McIntosh and Bonham-Smith, 2005) and environmental (Hulm et al., 2005) triggers.

1.3.3.1. R-protein gene regulation

Several prokaryotic, chloroplastic, and mitochondrial r-protein genes are organized in a similar manner despite some differences in their modes of expression. *E. coli* r-protein genes are arranged in operons, with expression under the control of a single operator (Mager, 1988), producing a single polycistronic mRNA that encode multiple proteins (Nomura et al., 1980). In *E. coli*, several r-proteins can regulate their own expression via a negative feedback system where an r-protein binds to its operon to inhibit further transcription that is referred to as autogenous regulation (Gregory et al., 1988). Autogenous regulation has been reported for RPL4, a primary binding r-protein, that can bind to its *S10* operon, impeding transcription by causing premature termination at the operon leader sequence (Zengel et al., 1994). As well, RPL4 plays a role in post-transcriptional regulation of the *S10* operon by binding to its polycistronic transcript thereby preventing translation (Fallon et al., 1979). In *E. coli*, the gene of another primary binding r-protein, *RPL20*, is transcribed as part of the

IF3 operon containing *IF3*, *RPL35* and *RPL20*. Depending on the amount of *RPL20* present in the cell (Chiaruttini et al., 1996), it can bind to its polycistronic mRNA transcript at two distinct regions to form a pseudoknot that represses translation of the transcript (Guillier et al., 2002; Guillier et al., 2005). Translational regulation has also been observed for the *spc* operon (Olins and Nomura, 1981), where r-protein *RPS8* can repress expression of the third cistron (that encodes *RPL5*) by binding the 3' end of the Shine-Dalgarno sequence of the cistron (Merianos et al., 2004). All of these mRNA binding sites are quite similar in sequence to the corresponding protein binding sites on the rRNAs (Nomura et al., 1980), with this mimicry being the basis for the negative feedback mechanism.

Similar mechanisms are thought to operate in chloroplasts. This prokaryotic type of r-protein gene organization appears to be conserved in chloroplasts and, to some degree, in mitochondria. Plastid r-protein genes are organized in “remnants” of prokaryotic operons. Few plant plastid genes contain introns (only 15 in tobacco) with 4 (*RPS12*, *RPS16*, *RPL2*, *RPL16*) of the 22 total r-protein genes contain 500-1000 bp introns (Shinozaki et al., 1986). In algae and higher plants, many of the r-protein genes that are present in prokaryotes, are absent in plastid r-protein operons (e.g. three genes within the *E. coli* *S10* operon are absent in these plant plastid genomes) due to nuclear genome transfer (Shinozaki et al., 1986). Plant plastid r-protein gene promoters are very similar to those found in *E. coli* with characteristic -10, -35 regions, and 100 bp upstream of their start codons are consensus Shine-Dalgarno-like ribosome binding site sequences (Bonham-Smith and Bourque, 1990). Additional prokaryotic gene similarities include plastid transcription terminator and ribosomal binding sites, which are essentially identical to those found in *E. coli* r-protein genes. This suggests plant plastid r-protein gene regulation is more likely related to that of prokaryotes than eukaryotes.

The organization of r-protein genes in lower plant mitochondrial genomes, such as liverworts, is similar to bacterial cistrons (Schuster and Brennicke, 1994). This is in contrast to the mitochondria in higher plant species, whose mitochondrial genomes contain fewer r-protein genes than plastid genomes. The Arabidopsis mitochondrial genome contains the *RPL2*, *RPL5*, *RPL16*, *RPS3*, *RPS4*, *RPS7*, and *RPS12* r-protein genes which lack Shine-Dalgarno sequences, a characteristic of all mitochondrial genes. Some plant mitochondrial r-protein genes are clustered, where clusters can contain as few as 2 (*RPS15-RPS14*) r-protein

genes, as seen in *Petunia*, to as many as many as 12 (*RPS10-RPS12-RPS19-RPS3-RPS16-RPL5-RPS14-RPS8-RPL6-RPS13-RPS11-RPS1*) as seen in liverworts. Angiosperms mitochondrial r-protein genes however tend to be less clustered with only partial conservation of the operon arrangement (likely due to recombination events), and more of their r-protein genes found scattered elsewhere in the nuclear genome (Gillham et al., 1994). In addition, several plant mitochondrial genes also contain introns, and it is hypothesized that multiple transcription factors are involved in transcriptional regulation of the various mitochondrial r-protein genes (Schuster and Brennicke, 1994). It is assumed that plant mitochondrial genes are transcribed as polycistronic mRNAs, although there is a lack of information surrounding higher plant mitochondrial gene regulation. Research suggests that plant mitochondrial gene promoters appear to be unique to plants and different from prokaryotes (Binder and Brennicke, 2003), proposing that mitochondrial r-protein genes are a mosaic of both prokaryotic and eukaryotic elements.

Feedback regulatory mechanisms for eukaryotic r-proteins do not appear to be as prevalent or well characterized as in prokaryotes. In yeast, the most prevalent mode of regulation occurs at the transcriptional level (Zhao et al., 2006), where repressor activator protein-1 (Rap1) binds to activator core sequences found within the promoters of the majority of r-protein genes (Tsang et al., 1990), to regulate their expression. The Rap1 binding site consists of a RPG box, HOMOL1, and UAS_{rp} (upstream activating sequence for r-protein genes; Woudt et al., 1987; Rotenburg and Woolford, 1986), that are required for transcription of yeast r-proteins, particularly *RPL25*, *RPS16a*, and *RPL16a* (Moehle and Hinnebusch, 1991). In yeast, there are several other proteins that interact with r-protein genes to regulate transcription (with the exception of *RPL3* and *RPS33* [Planta and Raue, 1988; Vignais et al., 1987]) e.g., translation upshift factor (TUF), a protein factor that binds to the conserved RPG-box. It appears that in yeast, the response in r-protein gene expression to nutritional changes is mediated by TUF (Powers and Walter, 1999). In *S. cerevisiae*, a silencer binding protein, Abf1, (Hardy et al., 1992) is multifunctional: it negatively regulates expression of *RPL2a* and *RPL2b* by binding to its silencer sequence in the promoters of these two genes (Della Seta et al., 1990) while it activates expression of r-proteins that do not contain RPG boxes (i.e. *RPS33* and *RPL45* contain different nucleotide motifs). It appears that the affinity for binding of Rap1 or Abf1 to r-protein gene promoters and the resulting

nucleosome remodeling is the main mechanism of regulation. In addition to transcriptional regulation, there is also evidence for post-transcriptional regulation of r-protein synthesis in yeast. R-protein RPS14 can bind to its pre-mRNA transcript (*RPS14B*) to prevent translation. Other yeast r-proteins, including RPL32 and RPL2, are also involved in post-transcriptional regulatory mechanisms (Fewell and Woolford, 1999). Mammalian r-protein RPS14 can also bind to its own transcript (Tasheva and Roufa, 1995) suggesting that a similar feedback regulatory mechanism may also operate in mammals.

Alternative splicing, where a pre-mRNA transcript is spliced to yield a different gene product than what is normally transcribed, may also play a role in mammalian r-protein gene regulation (Brett et al., 2002). R-protein transcripts of *Caenorhabditis elegans* (*C. elegans*) have been found to be alternatively spliced (Mitrovich and Anderson, 2000) and mRNA transcripts of human r-proteins RPL3 and RPL12 have been found to contain nonsense codons (amber [UAG], ochre [UAA] and opal [UGA]) after alternative splicing. Premature stop codons within mRNA result in nonsense mediated protein decay (Cuccurese et al., 2005). For example, in humans the level of RPL3 in cells was found to regulate the amount of alternative splicing of the RPL3 transcript (Cuccurese et al., 2005). Although alternative splicing of plant r-protein mRNAs has yet to be identified, it would be interesting to align plant *RPL3* and *RPL12* cDNAs with those of human, mouse and bovine species, as the alternatively spliced sequences appear to be highly conserved; nucleic acid identity ~90% between mammalian *RPL3* splice sequences.

Although promoter elements important in the regulation of expression of some r-protein gene in plants (e.g. RPL34; Dai et al., 1996) have been identified, the primary mechanism for plant r-protein gene regulation has yet to be determined. It is presumed that the expression of r-proteins is an organized process as several members of each r-protein gene family are actively transcribed. Stress and exogenous hormones appear to alter the level of r-protein gene transcription (McIntosh and Bonham-Smith, 2005; Hulm et al., 2005) however, the pathways by which this occurs have yet to be described.

1.4. R-protein-RNA binding

RNA specific protein recognition involves unique secondary and tertiary structural features of the RNA. The assembly of ribosomal subunits relies on the orderly association of

r-proteins with rRNA domains, following a hierarchical pathway involving different classes of r-proteins. It is thought that primary binding r-proteins bind rRNA independently, inducing conformation changes that facilitate the binding and sequential addition of another class of r-proteins, secondary binding r-proteins, and these facilitate the association of tertiary binding r-proteins. This hierarchical framework suggests that primary binding r-proteins are essential for ribosomal subunit assembly. It has been suggested that the structure of the RNA molecule is more important for protein recognition than the RNA sequence itself, as proteins can recognize and bind to RNA hairpins, bulges, internal and junction loops, and helices specifically (Draper, 1995). RPL11, for example, exemplifies this argument. RPL11 binding to large subunit RNA is not affected by sequence changes to its rRNA binding site, a helix of the GTPase center of 23S rRNA. RPL11 contacts the RNA backbone and recognizes only the 3-dimensional structure of the RNA molecule, and not specific bases (Blyn et al., 2000; Draper, 1995). Current literature suggests that r-proteins can recognize specific RNA structures via specific domain orientations. Specific α -helical orientations allow for suitable hydrogen bonding of the protein to the bases of rRNA, whereas β -sheets containing aromatic amino acids interact with unstacked RNA bases, allowing polar amino acids to hydrogen bond to RNA bases. The conformation of the RNA backbone also appears to play a role in r-protein-rRNA recognition and specificity of interactions (Draper and Reynaldo, 1999). Despite the overwhelming amount of information available on DNA-binding domains, RNA binding domains are less characterized, as are RNA-protein interactions.

1.4.1. R-protein-RNA binding strategies

Most r-proteins are conserved across kingdoms, with homologs existing between bacteria and eukaryotes (Yusupov et al., 2001). A number of r-proteins interact directly with rRNAs, and this binding is thought to be facilitated by the presence of at least three different nucleic acid binding motifs.

The assembly of ribosomal subunits and pre-rRNA maturation relies on specific RNA-protein interactions. R-protein RPL11, which has a RNA binding domain, referred to as RPL11-C76, and is a putative homeodomain-like- α -helical nucleic acid binding protein, is highly conserved throughout eubacteria, archaebacteria and eukarya (Draper and Reynaldo, 1999). These binding proteins are characterized by three α helices with a large loop between

the first and second helices and a β sheet present between helices 2 and 3 to link the termini together (Draper and Reynaldo, 1999). The RNA binding domain is located near the C-terminus of helix 3, and is strikingly similar to that of homeodomain proteins in which the highly conserved helix 3 hydrogen bonds to the major groove of DNA. RPL11 also contains a flexible loop region (amino acid residues 86–96, conserved throughout all domains of life) which is proposed to function as a clamp for the protein to “grasp” and hold RNA while the RNA binding domain interacts with the RNA surface (Shen et al., 1995).

Another binding motif, the OB motif is present in r-proteins RPS1 and RPS17 and is made up of amino acids T²¹, H⁴⁸, F⁷⁴, along with other amino acids found in a large loop structure in these r-proteins. The large loop structure, similar to that present in RPL11, is thought to clamp the RNA already bound at the OB fold. For RPS1, a putative 70 amino acid residue β -barrel-like RNA binding domain has been resolved by NMR (Bycroft et al., 1997). The binding site is actually six OB fold repeats that could function in a lengthened interaction with the RNA binding site. A third RNA binding motif, found in RNP motif proteins (i.e. RPS6) is comprised of two sequences, RNP1 and RNP2, which are separated by 30 amino acids. The motif is characterized by a series of β -strands ending with a highly disordered loop, which is believed to contact RNA lying in between the β 2- β 3 strands. Several other putative RNA binding domains exist in other r-proteins, such as an α - β - α - β - β fold found in the N-terminus of the prokaryotic RPS8, while the putative RNA binding domain of eukaryotic RPS15a is located at the C-terminus (Cerretti et al., 1988; Chang et al., 2005). Identifying and analyzing RNA-protein binding structures by NMR and X-ray crystallography will provide insight into the biological activity of the ribosome. Every primary binding r-protein makes contact with rRNA in some form, and this contact is independent of other r-proteins. The secondary structure of rRNA is critical for further r-protein recognition as different r-protein binding can span a number of different loops in the rRNA (Muto et al., 1974).

1.4.2. R-protein-rRNA binding

There is a high degree of conservation of structure between prokaryotic and eukaryotic rRNAs, suggesting that primary binding r-proteins (also highly conserved) exploit similar RNA binding sites. The confirmed C-terminal RNA binding domain of RPL11 from

the eubacterium *Bacillus stearothermophilus* (*B. stearothermophilus*), and the archaeobacterium *Sulfolobus acidocaldarius* (*S. acidocaldarius*) and eukarya (*S. cerevisiae*) contains highly conserved residues that interact with small subunit rRNA, indicating that the rRNA binding domain has been conserved throughout the course of evolution (Xing et al., 1997). In addition, evolutionarily conserved r-protein-rRNA interactions have also been confirmed between yeast and *E. coli*. Switching the rRNA binding domains between homologous r-proteins RPL11 from *E. coli* and RPL12 from yeast produced recombinant proteins that were still functional and able to bind their respective rRNAs (Thompson et al., 1993), highlighting a conserved interaction involving primary binding r-protein homologs. In yeast, during the initial assembly of the 60S subunit, RPL25 recognizes and binds to domain III of 25S rRNA (Stoldt et al., 1998). RPL25 contains two regions involved in binding to domain III of 25S rRNA, and mutational analysis has shown that a complete intact domain III is needed for proper 25S secondary and tertiary structure (van Beekvelt et al., 2000).

Mapping the assembly of the prokaryotic 30S subunit has provided a detailed picture of binding positions for RPS12, RPS15, RPS16, and RPS17 on 16S rRNA (Held et al., 1974). Furthermore, incubating the platform (or central domain, Figure 1.1) of 16S rRNA, comprised of nucleotides 547-895, with all of the 30S r-proteins identified putative primary binding proteins (Agalarov et al., 1998). All of the 30S primary binding proteins interact with the platform domain of 16S rRNA and localize to the side bulge of the 30S subunit (Davies et al., 1996). Primary binding RPS8 and RPS15 independently bind and associate with 16S rRNA, while secondary binding of RPS18 relies on RPS8 and RPS15 early binding. RPS8 initially binds to the platform domain of 16S rRNA through a series of contacts mapped to nucleotides 597-599/640-643 within helix 21 (Jagannathan and Culver, 2003). Specifically, two loops comprising C-terminal amino acids 86-93 and 115-116 interact with helix 21. However, site-directed mutagenesis of RPS8 has also implicated the N-terminus in rRNA binding. Hydroxyl radical footprinting, a technique that generates “footprints” of proteins bound to DNA or RNA, suggests the N-terminus interacts with helix 25, further stabilizing the central domain. NMR microscopy has provided greater detail to the RPS8-16S rRNA binding domains, and indicates the involvement of nucleotides G588 to G604 and C634 to C651 of 16S rRNA (Sun et al., 2004). Specifically, the RPS8 core rRNA binding

site relies on four nucleotides, 597/641/642/643 while non-core binding nucleotides 595/598/640 are also important (Kalurachchi et al., 1997) often requiring Mg^{2+} for complex stability.

1.4.3. R-protein-mRNA binding

Several ribosomal and non-ribosomal proteins interact with and bind to mRNA. R-proteins primarily of the small subunit, such as RPS1 (containing the largest RNA binding domain of all r-proteins), RPS7 and RPS11 play major roles interacting with mRNA transcripts during translation. Cryo-EM studies have identified several mRNA elements required for small subunit association in both eukaryotes and prokaryotes. As previously mentioned, in prokaryotes, RPS8 binds to regulatory sequence within its polycistronic transcript, more specifically, the 5' end of the third cistron (RPL5) of the *spc* operon mRNA (Gregory et al., 1988; Kalurachchi, 1997; Tishchenko et al., 2001). The mRNA regulatory sequence to which RPS8 binds is very similar to the 16S rRNA binding regions, indicating that RPS8 binds to similar rRNA and mRNA structures. Sequence analysis of the *spc* operon transcript suggests that it could possibly generate a similar secondary conformation to that of 16S rRNA (Mattheakis and Nomura 1988). However, the RPS8-mRNA interaction is approximately 5 times weaker than the RPS8-16S rRNA interaction. To date, there are no structural data for RPS8 bound to the *spc* polycistronic transcript. Structural analysis of this interaction would give further detail to changes in conformation of the mRNA upon RPS8 binding. Recently however, crystal structures of autogenous RNA representing the regulatory site of the *spc* polycistronic transcript, with RPS8 bound, have been obtained (Merianos et al., 2004). It was determined that RPS8 binds to three parts of the mRNA loop, consisting of nucleotides A12–G17, C34–C41 and U28–A31, that mimic the structure of helix 21 (Merianos et al., 2004). Interestingly, RPS8 binding to the internal loop of the *spc* polycistronic mRNA regulatory region mimics its interactions on helix 20/21/22 of 16S rRNA in *T. thermophilus* (Brodersen et al. 2002).

Eukaryotic r-proteins also interact with mRNA and pre-mRNA as a means to regulate translation. RPS14 and RPL23 of *S. cerevisiae* directly interact with specific regions on their mRNAs and pre-mRNA transcripts. RPS14 was the first eukaryotic r-protein shown to bind to both its mRNA transcript and 18S rRNA (Fewell and Woolford, 1999). Specifically in the

RPS14B transcript, the first exon and nucleotides from the adjacent intron form a RNA stem loop that is recognized by its r-protein RPS14 (Fewell and Woolford, 1999). This provides evidence, similarly to mechanisms in prokaryotes, that secondary structures formed on mRNA serve as a means for post-transcriptional regulation. Mutations in the putative RNA binding domains of RPS14 resulted in lower affinity binding to both its rRNA and mRNA targets. It is likely that other eukaryotic r-proteins will also bind to their mRNA transcript as a form of post-transcriptional repression, although other more complex modes of regulation also likely exist (as discussed in section 1.3.3.1).

1.5. R-protein S15a

The proposed research focused on the Arabidopsis RPS15a multi-gene family, comprised of six members: *RPS15aA-F*. The first Arabidopsis *RPS15a* gene (*AtRPS15a*) was identified by Bonham-Smith and Moloney (1994) by screening the Arabidopsis genome with a *Brassica napus* (cv Westar) S15a cDNA. This particular r-protein family (S8p, based on prokaryotic ortholog) has been shown to interact with small subunit rRNA, particularly at the body structure and central domain of the 30S subunit. Although no crystal structures exist for RPS15a specifically, it is possible that it contains similar domain contacts to its prokaryotic ortholog, RPS8. The entire S8p family belongs to evolutionary group I, with orthologs found in eukarya, archaeobacteria, and eubacteria. The deduced Arabidopsis RPS15a isoforms are approximately 15 kDa in size with a narrow range of pI values, between 10-10.8, owing to the high basic amino acid content of this particular r-protein. RPS15aB and RPS15aE are evolutionarily distinct (claded as type II) from the other four RPS15a cytosolic isoforms, RPS15aA, RPS15aC, RPS15aD and RPS15aF (claded as type I). The two types of RPS15a proteins are slightly different in their abundance and properties. Type I RPS15a proteins are more basic and found in higher quantity than type II RPS15a proteins, as determined by MS analysis of Arabidopsis ribosomal pellets (Chang et al., 2005). Type II RPS15a isoforms are thought to be targeted and incorporated into mitochondrial ribosomes (Adams et al., 2002). It has been suggested that these two RPS15a isoforms replace the mitochondrial RPS8 ortholog, as the mitochondrial and nuclear genomes of several flowering plants have lost the ancestral eubacterial *RPS8* (Adams et al., 2002). In fact, in all angiosperms, where sequence is available, the ancestral eubacterial *RPS8*

mitochondrial gene is absent, and therefore it is plausible to suggest that the original eukaryotic cytosolic counterpart, RPS15aB and or RPS15aE, accommodate this loss. All copies of the Arabidopsis RPS15a gene family appear to be expressed except for RPS15aC, with RPS15aA, RPS15aD, and RPS15aF mRNA transcripts being most abundant in mitotically active tissues (Hulm et al., 2005).

1.6. Objectives

The experiments described in this thesis investigated the localization and binding characteristics of the putative primary binding, six-member *RPS15a* gene family of *Arabidopsis thaliana*. Specific objectives were to:

1. Characterize the subcellular localization of type II RPS15aB and RPS15aE.
2. Identify whether RPS15aA/F, -D, -E interact with cytoplasmic and/or mitochondrial 18S rRNA.
3. Determine whether RPS15aA/F, -D, -E bind three specific 18S rRNA helix 23 loop fragments.
4. Determine whether RPS15aA/F, -D, -E bind their respective mRNA transcripts.
5. Identify whether RPS15aA/F, -D, -E can bind to single stranded or double stranded DNA sequence corresponding to helix 23 of 18S rRNA.

CHAPTER 2. MATERIALS AND METHODS

2.1. Plant material

Arabidopsis thaliana (*A. thaliana*) cultivar Columbia-0 was used in the experiments outlined below. Four-week old tobacco (*Nicotiana tabacum*) cultivar Petit Havana was also used in the confocal experiments outlined below. All plants were grown under a 23°C/18°C temperature regime and a 16 h-light/8 h-dark photoperiod of $\sim 120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

2.2. Strains and selective media

Escherichia coli (*E. coli*) Tuner cells (Novagen, EMD Biosciences, Darmstadt, Germany) were used to host the protein expression plasmid, pBI784 (National Research Council-Plant Biotechnology Institute). Tuner strains are *lacZY* deletion mutants of *E. coli* BL21 and enable adjustable levels of protein expression throughout all cells in a culture. The *lac* permease (*lacY*) mutation allows uniform entry of isopropyl β -D-1-thiogalactopyranoside (IPTG) into all cells in the population, which produces a concentration-dependent, homogeneous level of induction. *Agrobacterium tumefaciens* (*A. tumefaciens*) strain pC2760 (Hoekema et al., 1983) were used to host fluorescent protein constructs in the binary vectors pGREEN10029 and pCAMBIA 1381 (Hellens et al., 2000) whereas the GV3101 strain of *A. tumefaciens* was used to host pBINmgfp5- β -ATPase (Logan and Leaver, 2000). *E. coli* strains DH5 α and MC1061 were used to host all other plasmid constructs. All of the strains were grown in 50 $\mu\text{g/mL}$ ampicillin or 25 $\mu\text{g/mL}$ kanamycin supplemented media, prepared according to Sambrook et al. (1989), with all incubations at 37°C.

2.3. Construct cloning

Standard molecular cloning techniques as described in Sambrook et al. (1989) were followed for the generation of all constructs. All putative clones were confirmed via automated sequencing (National Research Council-Plant Biotechnology Institute, Saskatoon,

SK). Restriction enzymes, Pfu DNA polymerase, reverse transcriptase, and T4 DNA ligase were obtained from Fermentas or Invitrogen (Burlington, Ontario). All histidine-tagged constructs in pBI784 were used to transform *E. coli* Tuner cells. Cells were grown in Luria-Bertani broth (LB) media containing 50 µg/mL ampicillin, which was prepared according to Sambrook et al. (1989), and incubations were carried out at 37°C. Plasmid DNA concentration was determined by the GeneQuant II (Pharmacia Biotech, Cambridge, U.K.) DNA calculator.

2.4. Fluorescent RPS15aB and RPS15aE constructs

Arabidopsis bud tissue was collected and stored at -80°C prior to RNA isolation. Bud tissue was ground in liquid nitrogen to a fine powder prior to the addition of extraction buffers as directed in the manual of the RNeasy Plant Mini kit (Qiagen, Valencia, CA). Total RNA was treated with RNase-free DNase I (Fermentas), following the manufacturer's instructions. C-terminal monomeric RFP (mRFP) RPS15aB/-E proteins were obtained by amplifying RPS15aB and RPS15aE open reading frames (ORFs), without stop codons (from cDNA), from total bud RNA, extracted from 100 mg of Arabidopsis seedling tissue using a RNeasy Plant Mini kit (Qiagen). ORFs were amplified using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) following the manufacturer's instructions with one modification to the protocol: template RNA was incubated with the gene specific reverse primers, RVS RPS15aB and RVS RPS15aE (Table 2.1), for 40 min at 48°C. The subsequent first strand product was used as template for polymerase chain reaction (PCR) amplification using Pfu DNA polymerase (Fermentas). The type II RPS15a ORFs were amplified from 2 µL of first strand product with gene specific forward (FWD RPS15aB and FWD RPS15aE, Table 2.1) and reverse (RVS RPS15aB and RVS RPS15aE, Table 2.1) primers and Pfu DNA polymerase (Fermentas). PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. Appropriate DNA fragments were excised and purified with a PCR purification kit (RBC Bioscience, Taipei, Taiwan). The PCR products were directly cloned into unique *EcoRI/BamHI* restriction sites in pGREENI0029 (Hellens et al., 2000), a binary vector containing a modified multiple cloning site including a tandem repeat of the *Cauliflower mosaic virus* (CaMV) 35S promoter (35S), a glutathione-S-transferase (GST)

Gene Name	Primer Name	Primer Sequence (5' – 3')	Fragment Amplified)	Estimated size # (bp)
<i>RPS15aB</i>	FWD RPS15aB	GCGGAATTCATGGGGAGGAGAATATTG	5'-EcoRI- RPS15aB- BamHI-3'	387
	RVS RPS15aB	GCGGGATCCATGAAAGAAACCAAG		
<i>RPS15aE</i>	FWD RPS15aE	GCGTCGACCATGGGGAGGAGGATTTTG	5'-EcoRI- RPS15aE- BamHI-3'	387
	RVS RPS15aE	GCGGGATCCGTAAAAGAAGCCAAGA		
<i>RPS15aD</i>	5'RPS5aD ORF	GCGTCGACCATGGTGAGAATCAGTGTGCTC	5'SalI- RPS15aD- NotI 3'	393
	3'RPS15aD ORF	CGCGCGGCCGCATCAGTAAAAGAACCCAAG		
<i>RPS15aE</i>	5'RPS15aE ORF	GCGTCGACCATGGGGAGGAGGATTTTG	5'SalI- RPS15aE- NotI3'	390
	3'RPS15aE ORF	GCGGCGGCCGCTCAGTAAAAGAAGCCAAGA		
<i>RPS15aF</i>	5'RPS15aF ORF	GCGTCGACCATGGTGAGAA TCAGTGTGC	5'SalI- RPS15aF- NotI 3'	393
	3'RPS15aF ORF	CGCGCGGCCGCATCAGTAAAAGAAGCCAAG		

Table 2.1. Oligonucleotide primers used to amplify fragments of various *RPS15a* genes for molecular cloning.

tag, mRFP, and a nopaline synthase (nos) poly(A) signal (terminator) creating the fusion proteins RPS15aB/-E-GST-mRFP under the control of a tandem 35S promoter (Degenhardt and Bonham-Smith, 2008, Figure 2.1 A).

2.5. Histidine-tagged constructs in pBI784

Total Arabidopsis bud RNA was extracted as described above. ORFs for RPS15aA/F,-D,-E were amplified using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) following the manufacturer's instructions with one modification to the protocol: template RNA was incubated with gene specific reverse primers (3'RPS15aD ORF; 3'RPS15aE ORF; and 3'RPS15AF ORF, Table 2.1) for 40 min at 48°C. The subsequent first strand products were used as template for PCR amplifications via Pfu DNA polymerase (Fermentas). Specifically, 2 µL of first strand template was incubated with gene specific forward (5'RPS15aD ORF; 5'RPS15aE ORF; and 5'RPS15AF ORF, Table 2.1) and reverse (3'RPS15aD ORF; 3'RPS15aE ORF; and 3'RPS15AF ORF, Table 2.1) primers and 1.24 units of Pfu DNA polymerase. PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and DNA fragments of the appropriate size were excised and purified with a PCR purification kit (RBC Bioscience). Clean PCR products were cloned into unique *SalI/NotI* restriction sites in pBI784, which includes an inducible *lac* promoter 5' to the T₇ RNA polymerase gene (Figure 2.1 B). Therefore, with IPTG induction, T₇ RNA polymerase is transcribed, resulting in recombinant protein expression.

2.6. 18S rDNA and RPS15a cDNA fragments in pBluescript II KS+

Full length cytoplasmic 18S rDNA, mitochondrial 18S rDNA, and individual helix 23 (cytoplasmic rDNA) loop fragments were amplified by PCR from total Arabidopsis cellular DNA. Briefly, total Arabidopsis cellular DNA was isolated from 100 mg of flash frozen Arabidopsis bud tissue, ground into a fine powder, following the E.Z.N.A Plant DNA Miniprep kit manufacturer's protocol (Omega Bio-tek, Doraville, GA). rDNA and cDNA were amplified from 100 ng of template DNA, using gene specific forward primers (5'18S rRNA, 5'Mit18S rRNA, 5'18S rRNA H23 loop 1-6, 5'18S rRNA H23 loop 1-2, 5'18S rRNA H23 loop 4-6, Table 2.2), gene specific reverse primers (3'18S rRNA, 3'Mit18S

A)



———— = 307 bp

B)



———— = 55 bp

Figure 2.1. RPS15a fusion protein cassettes. A) Fluorescent protein fusion constructs in the vector pGREEN0029. *CamV* 35S x 2, a cauliflower mosaic virus (CaMV) 35S promoter; *RPS15a*, the *RPS15aB/-E* ORF minus a stop codon; GST, a glutathione-S-transferase tag, mRFP, a monomeric red fluorescent protein tag; *nos*, a nopaline synthase poly(A) signal (terminator) (Degenhardt and Bonham-Smith, 2008). B) Histidine fusion protein construct in the protein expression vector pBI784. T₇, a T₇ promoter; H, a tandem repeat of 6 histidine residues; *RPS15a* ORF, the open reading frame of *RPS15a*; Terminator, a T₇ transcription terminator sequences.

Gene Name	Primer Name	Oligonucleotide Sequence (5' - 3')	Fragment Amplified	Estimated # size
<i>At 18S rRNA</i>	5'18S rDNA	GCGAATTCAAACAAGGCTCAAGCATTGAAA	5'SpeI - 18S rDNA-PstI 3'	1808 bp
	3'18S rDNA	GCGCTGCAGCAATGATCCTTCCGCAGGTTCA		
<i>At Mit 18S rRNA</i>	5'Mit18S rDNA	GCGAGCTCATCATAGTCAAAAGAAGAGTTTGAT	5'SacI-mit 18S rDNA-XbaI 3'	1935 bp
	3'Mit18S rDNA	GCGTCTAGAGGATTCAATCCAGCCACAGGTTCCC		
<i>At 18S rRNA</i> nt: 645-858	5'18S rDNA H23 loop 1-6	GCGGAGCTC GGGATGGGTCGGCCGGTC	5'SacI-18S rDNA helix 23-XbaI 3'	213 bp
	3'18S rDNA H23 loop 1-6	GCGTCTAGAACTGCGATCCCCAAGGCC		
<i>At 18S rRNA</i> nt: 645-738	5'18S rDNA H23 loop 1-2	GCGGAGCTC GGGATGGGTCGGCCGGTC	5'SacI-18S rDNA helix 23-XbaI 3'	94 bp
	3'18S rDNA H23 loop 1-2	GCGTCTAGACGCCGGAGGCACGACCCG		
<i>At 18S rDNA</i> nt: 739-858	5'18S rDNA H23 loop 4-6	GCGGAGCTCTCCGGCGCTGTTACTTTG	5'SacI-18S rDNA helix 23-XbaI 3'	119 bp
	3'18S rDNA H23 loop 4-6	GCGTCTAGAACTGCGATCCCCAAGGCC		
<i>RPS15aD</i>	5'RPS15AD-cDNA	GCGAAGCTTGACTAAAATGAGTAAATGCCACA	5'HindIII-RPS15aD cDNA-NotI 3'	743 bp
	3'RPS15AD-cDNA	GCGAATTCGACTAAAATGAGTAAATGCCAC		
<i>RPS15aE</i>	5'RPS15AE-cDNA	GCGCTGCAGCCGATTTGTGTAGTATCGTCG	5'PstI-RPS15aE cDNA-EcoRI 3'	668 bp
	3'RPS15AE-cDNA	GCGAATTCATAGAAGAAATATAGATTATTC		
<i>RPS15aF</i>	5'RPS15AF-cDNA	GCGCTGCAGAATTCGCATCTTGTATTAGT	Not cloned	803 bp
	3'RPS15AF-cDNA	GCGACTAGTTACCTGGTTGATCCTGCCAGTA		

Table 2.2. Oligonucleotide primers used in PCR amplifications of rDNA and *RPS15aD/-E* cDNAs.

rRNA, 3'18S rRNA H23 loop 1-6, 3'18S rRNA H23 loop 1-2, 3'18S rRNA H23 loop 4-6, Table 2.2) and Pfu DNA polymerase (Fermentas). The full length cytoplasmic 18S rDNA PCR product was cloned into the unique restriction sites *SpeI/PstI* of pBluescript II KS+ (pBSKS+) (Stratagene, La Jolla, CA), whereas full length mitochondrial 18S rDNA was cloned into the unique *SacI/XbaI* of pBSKS+. All helix 23 loop 1-6, 1-2, and 4-6 PCR products were cloned into the unique restriction sites *SacI/XbaI* in pBSKS+. Full length *RPS15aD* and *RPS15aE* cDNAs were obtained by RT-PCR amplification from Arabidopsis total bud RNA (section 2.5). *RPS15aD* and *RPS15aE* full length cDNAs were amplified using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) following the manufacturer's instructions with one modification: template RNA was incubated with gene specific reverse primer (3'RPS15aD-cDNA, 3'RPS15aE-cDNA, Table 2.2) for 40 min at 48°C. The subsequent first strand product was used as template for PCR amplification via Pfu DNA polymerase (Fermentas). Specifically, 2 µL of first strand template was incubated with gene specific forward (5'RPS15aD-cDNA, 5'RPS15aE-cDNA, Table 2.2) and reverse (3'RPS15aD-cDNA, 3'RPS15aE-cDNA, Table 2.2) primers and 1.24 units of Pfu DNA polymerase. The *RPS15aD* full length PCR fragment was cloned into the unique *HindIII/EcoRI* restriction sites of pBSKS+ whereas *RPS15aE* full length cDNA PCR product was cloned into the unique restriction sites *PstI/EcoRI* of pBSKS+.

2.7. Expression of histidine-tagged *RPS15a*

RPS15a proteins were expressed in *E. coli* Tuner cells closely following the QIAexpress System (Qiagen). Briefly, 4 mL of LB medium containing 50 µg/mL ampicillin was inoculated with *E. coli* Tuner cells containing each of the 6x-histidine-tagged *RPS15a* constructs within the pBI784 vector. Cultures were grown overnight at 37°C with shaking. Fresh pre-warmed media (250 mL containing 50 µg/mL ampicillin) was inoculated with 2 mL of an overnight culture. Cultures were further incubated with shaking at 37°C to an OD₆₀₀ ~0.8, when IPTG was added to a final concentration of 1 mM and cultures were left to shake at 37°C while hourly samples were taken 1-5 h after IPTG induction. Cells were harvested by centrifugation at 4°C for 20 min at 4000 g. The cell pellet was washed with ultra pure water, frozen in liquid nitrogen, and stored at -80°C. Negative controls were uninduced *E. coli* Tuner cell cultures harboring the histidine-tagged constructs.

2.8. RPS15a isoform extraction and purification

Two mg of each cell pellet was resuspended in 4 mL of non-denaturing lysis buffer (50 mM NaPO₄, 300 mM NaCl, pH 8.0) and sonicated (VirSonic, Vir Tis, Gardiner, NY) on ice for 6 x 10 s with 10 s pauses at 300 W. The sonicated cell lysate was centrifuged at 10,000 g at 4°C for 30 min, producing a cell debris pellet (crude fraction), and supernatant. The supernatant was decanted and stored at 4°C in preparation for purification, whereas the cell debris was stored at -20°C until further analysis. Prior to purification, and in order to determine the solubility of the recombinant RPS15a proteins, the supernatants and cell pellets were analyzed by 1D sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, section 2.9). Detectable amounts of RPS15a isoforms were found in the supernatant, and therefore the RPS15a isoforms were obtained under non-denaturing conditions.

Recombinant protein purification followed the “Batch purification of 6xHis-tagged proteins from *E. coli* under native conditions” protocol (QIAexpressionist handbook, 5th Ed., 2003, Qiagen). Four mL of the sonicated supernatant was incubated with 1 mL of 50% nickel-nitrilotriacetic acid (Ni-NTA) slurry (Qiagen) on ice with constant shaking at 200 rpm. Following the 60 min incubation time, fresh lysates containing the 50% Ni-NTA slurry (Qiagen) were loaded onto chromatography columns (Biorad, Hercules, CA) with the bottom outlet capped. Following manufacturer’s instructions, column flow-through, wash fractions, and finally, the nickel-affinity purified 6x-histidine tagged RPS15a protein elutions (all fractions pooled in 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0) were collected on ice. Purified proteins were stored in 50% glycerol at -20°C in preparation for electrophoretic shift assays (EMSAs). Protein concentrations of crude extracts and purified protein fractions were determined with the BCA™ Protein assay kit according to the manufacturer’s instructions (Novagen) with purified (bovine serum albumin) BSA as the control.

2.9. SDS-PAGE and Western blots

Protein expression and purification were analyzed by 1D SDS-PAGE. A Mini Protean III system (Biorad) was used to carry out SDS-PAGE following Sambrook et al. (1989) procedures. Crude cell debris and soluble fractions were isolated (as described above) and separated through 12% SDS-PAGs, stained with Coomassie Blue (Biorad) and visualized

using a digital camera. Protein purification was also analyzed by Western blotting (following SDS-PAGE) where separated proteins were transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA) using a Mini Trans-Blot Cell (Biorad) according to Sambrook et al. (1989). Dry membranes were blocked in phosphate buffered saline (PBS) with 5% milk for 1 h, and probed with a 1:200 dilution of a rabbit polyclonal 6x-histidine antibody (Santa Cruz Biotechnology Inc.), and washed in PBS. A 1:1500 dilution of peroxidase conjugated anti rabbit IgG [goat] secondary antibody (Rockland) was used to visualize 6x-histidine-tagged RPS15a proteins via an enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ).

2.10. In vitro transcription

RNA was synthesized via *in vitro* transcription from recombinant DNA templates. Specifically, pBSKS+ containing full length cytoplasmic 18S rDNA and mitochondrial 18S rDNA templates were linearized in preparation for *in vitro* transcription using T₇ RNA polymerase (Promega, Madison, WI) with the 5' cutting restriction endonucleases *Xho*I (cytoplasmic 18S rDNA) and *Eco*RI (mitochondrial 18S rDNA). 18S rRNA helix 23 fragment rDNA constructs were linearized with *Hind*III (Fermentas), full length *RPS15aD* cDNA was linearized with the restriction endonuclease *Sal*I (Fermentas) and full length *RPS15aE* cDNA was linearized with *Xho*I. Complete linearization of plasmids was confirmed on 1% agarose gels. Digested full length cDNA templates (1 µg) were *in vitro* transcribed by T₇ RNA polymerase (Fermentas) according to manufacturer's instructions, in the presence or absence of a Ribo m⁷G cap analog (Promega). Transcription reactions were conducted at 37°C for 2 h. Cytoplasmic and mitochondrial 18S rDNA as well as cytoplasmic 18S rRNA helix 23 rDNA fragments were *in vitro* transcribed using T₇ RNA polymerase from the RiboMAX™ Large Scale RNA Production System-T₇ kit (Promega). Transcription reactions were carried out at 37°C for 30 min. All *in vitro* transcription products were treated with 1 unit of RQ1 RNase-free DNase I (Promega) for 15 min to digest the DNA template. DNase I and RNA polymerase were extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and the transcribed RNA was precipitated with 95% ethanol. Unincorporated nucleotides were removed with a nucleotide removal kit (Qiagen) and transcription products were stored in 30 µL of diethyl pyrocarbonate (DEPC)

treated water. RNA concentrations were determined using a GeneQuant II (Pharmacia Biotech). Each *in vitro* transcription product (200 ng) was heated to 70°C and separated on a 1% agarose gel (containing ethidium bromide) to ensure only one correctly sized (relative to a high range RNA ladder, Fermentas) RNA band was present from each *in vitro* transcription reaction.

2.11. Preparation of single stranded and double stranded DNA

In order to determine whether RPS15a isoforms bind their single stranded rDNA sequences, linear PCR was used to amplify either the template or non-template strands of rDNA. PCR amplifications were carried out with Pfu DNA polymerase following manufacturer's directions (Fermentas) with one change to the standard protocol. Each reaction was performed with either the forward primer (5' 18S rRNA H23 loop 1-6, Table 2.2) or only the reverse primer (3' 18S rRNA H23 loop 1-6, Table 2.2) only to amplify single stranded DNAs from the template or non template strands, respectively. For double-stranded DNA products, an equal ratio of reverse and forward primers was used to amplify the 18S rRNA helix 23 loop 1-6 fragment from its pBSKS+ construct (section 2.6). All PCR products were separated on a 2% agarose gel followed by staining in ethidium bromide, and excision and extraction using the Qiaquick gel extraction kit (Qiagen). Double and single stranded DNA samples were eluted in 40 µL of DEPC-treated water and stored at -20°C in preparation for EMSAs.

2.12. Non-radioactive electrophoretic shift assays (EMSAs)

EMSAs were employed to identify RNA-RPS15a interactions. The technique is based on the principle that a complex of RPS15a protein and RNA will migrate through a non-denaturing agarose gel more slowly than free or unbound RNA molecules. Because the RNA templates used in the binding assays are much larger than those traditionally used, they will likely contain more non-specific binding sites and yield much smaller mobility shifts upon protein binding, due to the high degree of secondary structure of RNA, similar to the supercoil effect observed in DNA (Fried and Garner, 1998). RNA samples were pre-incubated in binding buffer (20 mM Tris-HCl [pH 8.5 at 37°C], 20 mM MgCl₂, 200 mM KCl) with 10 units of recombinant RNase inhibitors (Ribolock, Fermentas) at 37°C for 15

min prior to the addition of various amounts of RPS15a or BSA for a final volume of 10 μ L. Specifically, RPS15a isoforms were combined with: full length rRNAs (molar ratios of 131 : 1 - 250 : 1), rRNA helices (molar ratios of 4 : 1 - 15 : 1), mRNAs (molar ratios of 1.5 : 1 - 9.5 : 1), ssDNA (molar ratios of 6.8 : 1 - 18 : 1), dsDNA (molar ratios of 20 : 1 - 53 : 1). It should be noted that a variety of binding conditions were tested with varying salt concentrations, pH, and sample buffers that come close to approximating the physiology of the plant cell environment. All RPS15a isoforms were also incubated with a random RNA, provided as a control in the RiboMAX™ Large Scale RNA Production System-T₇ kit (Promega). Protein-RNA reaction mixtures were equilibrated for an additional 15 min at 37°C followed by a cool down for 10 min at 2°C. Two μ L of the 6x EMSA loading dye (Invitrogen) was then added to each mixture and each was immediately loaded onto a 1% agarose gel or a non-denaturing 3.5% polyacrylamide gel (for RNA loop fragment assays only). Gel electrophoresis was performed under non-denaturing conditions in 1% TAE buffer at 60 V for 1 h. Following electrophoresis, gels were stained in the dark with SYBR Green (Invitrogen, maximally excited at 495 nm, with a secondary excitation peak at 255 nm and when bound to RNA, an emission at 495/520 nm) for 20 min with gentle shaking and RNA bands were visualized with a Chemi3 darkroom (GE Healthcare, Piscataway, NJ) and all figures were prepared using Adobe Photoshop 7.0 software. Following staining with SYBR Green, polyacrylamide gels were rinsed with water and further stained in the dark with SYPRO Ruby (Invitrogen, maximally excited at 450 nm with a secondary excitation peak at 280 nm, when bound to protein, fluorescence emission is at 450/610 nm) for 3 h with gentle shaking. Excess SYPRO ruby dye was removed by washing with 10% methanol, 7% acetic acid for 1 h. Proteins were visualized and documented with the Chemi3 darkroom.

2.13. RNA competition assays

In order to analyze the specificity of cytoplasmic 18S rRNA loop fragment binding reactions, competition assays were conducted in which an excess of RPS15aE mRNA was added to the binding reactions. Competing nucleic acids at high concentrations, regardless of whether they are specific or non-specific, can reduce the specific binding of a protein to a target nucleic acid. If RPS15a binds specifically to the target 18S rRNA fragment, the semi-specific competing mRNA molecules should not reduce the complexed shift. Increasing

amounts of RPS15aD or RPS15aE mRNA were added simultaneously to the RNP assembly reaction mixture containing 100 ng of 18S rRNA helix 23 loop fragments. RPS15aD or RPS15aE mRNA and the RNA fragments were combined in ratios of 15:1 to 5:1. Following incubation, the reaction mixture was separated through a 1% agarose gel and the electrophoretic patterns were visualized by fluorescence imaging as described above (section 2.12).

2.14. Supershift assays

Antibody supershift assays were used to confirm histidine-tagged RPS15a proteins present in the protein/RNA complexes. An antibody in solution can aid in the detection of a shift, or it can block protein-RNA interactions thus diminishing a shift. If the anti-6x-histidine antibody binds RPS15a-RNA complexes, a supershift will result due to a further reduction in mobility for the RPS15a-RNA-antibody complex. Supershift assays were conducted under the same experimental agarose gel mobility shift assay conditions as detailed in section 2.12, with one difference. An anti-6x-histidine antibody, or an anti-RFP antibody (negative control, tested at .25, .5 and 1 µg) were incubated with the RPS15a-RNA complex for 30 min on ice after RPS15a-RNA complex formation. Three different concentrations of the anti-6x-histidine antibody, 0.25, 0.5 and 1 µg per binding reaction, were used to determine which concentration of antibody, if any, would produce a supershift.

2.15. Amino acid sequence alignments

The *A. thaliana* RPS15aB sequence (translated from coding sequence (CDS) NM_127530) RPS15aC (translated from CDS sequence NM_129517), RPS15aD (translated from TAIR accession # 2075230), RPS15aE (translated from CDS sequence NM_119088), and RPS15aF (translated from CDS sequence NM_125378) were compared to several orthologs: *Saccharomyces cerevisiae* RPS22A (accession # NP_012345), *Homo sapiens* RPS15a (ACCESSION # NP_001010), *Rattus norvegicus* RPS15a (accession # NM_053982.1), *Chlamydomonas incerta* RPS15a (accession # ABA01098), *Oryza sativa* putative RPS15a (accession # NP_001046846), *Physcomitrella patens* RPS15a (XP_001763465), *Populus trichocarpa* putative RPS15a (accession # ABK92904) and *Vitis vinifera* putative RPS15a (accession # CAN65143) using clustalW2 alignment software

provided at the European Bioinformatics Institute
(www.ebi.ac.uk/Tools/clustalw2/index.html).

2.16. Transient expression of RPS15aB and RPS15aE in tobacco

Binary vectors containing fluorescent protein constructs were used to transform *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) via electroporation using an ECM 399 electroporation system (BTX, Genetronic, San Diego, CA). RPS15aB and RPS15aE constructs in pGREENI0029 (Hellens et al., 2000) were co-electroporated with pSOUP to ensure pGREEN replication in *A. tumefaciens* as pSOUP provides replication factors in trans for pGREEN. Four-week-old tobacco plants were used for Agrobacterium-mediated transient expression. *A. tumefaciens* transformed with pGREENI0029-RPS15aB/-E-GST-mRFP, pCAMBIA1380-FIB2-EGFP (Degenhardt and Bonham-Smith, 2008) a nucleolar marker, and pBINmgfp5- β -ATPase (Logan and Leaver, 2000) a mitochondrial marker, were cultured at 28°C for 24 h, washed twice with infiltration buffer (50 mM MES [2-(N-morpholine)-ethane sulphonic acid] buffer pH 5.6, 0.5% glucose [w/v], 2 mM Na₃PO₄, 100 μ M acetosyringone from 200 mM stock in dimethyl sulfoxide), and resuspended in infiltration buffer, to an absorbance of 0.2- 0.3 at 600 nm. Using a 1 mL syringe (with no needle) tobacco leaves were infiltrated by mild pressure through the stomata of the lower leaf epidermal surface. Following infiltrations, tobacco plants were grown for 48-72 h in growth chambers in preparation for live cell imaging (section 2.17).

2.17. Confocal microscopy

Fluorescent images of RPS15aB/-E-mRFP proteins in tobacco leaf epidermal cells were obtained from an inverted Zeiss laser scanning microscopy 510 META confocal microscope (Jena, Germany). Post-infiltrated tobacco leaves (72 h) were cut into small squares (1 cm²) and mounted in water under a cover glass and examined under a 63x water immersion objective. Fluorescent images were photographed with a Zeiss MC63 camera, processed with Zeiss LSM Image Browser software, and prepared using Adobe Photoshop 7.0 software. Co-expression of GFP5- β -ATPase + RPS15aB/E-mRFP or AtFIBRILLARIN2 (FIB2)-GFP + RPS15aB/E-mRFP was attained with a line-switching multi-track option scanning mode. The multi-track option switches between excitation lines of an argon laser of

458/488 nm for GFP and a helium neon ion laser of 543 nm for RFP. Fluorescence from β -GFP5- β -ATPase and FIB2-EGFP was detected using a 515 nm dichroic beam splitter with 505-530 nm bandpass filter and 585-615 nm bandpass filter for RPS15aB/E-RFP. These specific settings (Brandizzi et al., 2003) prevented bleed-through of fluorescence. For imaging of the controls GFP5- β -ATPase and FIB2-EGFP, or RPS15aB/E alone, the same settings were used as described above.

CHAPTER 3. RESULTS

3.1. *RPS15a isoform sequence comparison*

The Arabidopsis RPS15a isoforms are encoded by a gene family consisting of 6 members distributed across chromosomes 1 through 5 [RPS15aA (1), RPS15aB (2) RPS15aC (2) RPS15aD (3), RPS15aE (4) and RPS15aF (5)]. Using needle pairwise alignment algorithms provided at the European Molecular Biology Laboratory-Bioinformatics Institute (<http://www.ebi.ac.uk/Tools/emboss/align/>), similarities in open reading frame (ORF) and polypeptide sequence identity of the RPS15a were identified (Table 3.1). Specifically, RPS15aB and -E differ from their eukaryotic cytoplasmic orthologs at the N-terminal domain, where the S22-like confirmed nuclear localization signal (NLS: GKRQVLIRP [Timmers et al., 1999] highlighted in gray, Figure 3.1, found only in type I RPS15as and other eukaryotic orthologs) lies. Additionally, only type I RPS15as contain N-terminal extensions, where in *E. coli*, greater than one-third of the 54 r-proteins possess protruding N-terminal extensions which make extensive contacts with rRNA (Guillier et al., 2005). An N-terminal extension is absent in both type II RPS15aB and RPS15aE and their prokaryotic counterpart, RPS8. Interestingly, the majority of the differences between type I and type II RPS15as reside within the first 40 amino acids of the N-terminus (compare yellow and blue highlighted amino acids in Figure 3.1). The type II r-protein, RPS15aB, contains a monopartite NLS sequence ‘KRGK’ (also a putative nucleolar localization signal) that is present in animal and plant type I RPS15a orthologs (Figure 3.1). Unlike the N-terminal domain, there is a high degree of amino acid sequence identity at the C-terminal domain, the putative small subunit rRNA binding region. Conservation at the C-terminus suggests RNA binding is conserved, and that the RPS15a isoforms likely bind similar RNA targets. Specifically, at the C-terminus, RPS15aA/F and RPS15aE share 70% identity whereas RPS15aD and RPS15aE share 63% amino acid identity. An interesting feature of the putative rRNA binding domain is the (S/T)-T-(S/T/P)-X-G motif located at the “ $\beta 6$ - $\beta 7$ ” turn in RPS8 (Tishchenko et al., 2001), where the fifth position glycine is conserved and

Gene	<i>RPS15aA</i>	<i>RPS15aB</i>	<i>RPS15aE</i>
<i>RPS15aA</i>	ORF Peptide	57% 53%	60% 54%
<i>RPS15aC</i>	77% 88%	54% 47%	55% 48%
<i>RPS15aD</i>	84% 98%	58% 51%	57% 52%
<i>RPS15aF</i>	85% 100%	57% 53%	57% 54%

Table 3.1. Predicted open reading frame (nucleotide sequence) and putative polypeptide sequence identity among the evolutionary divergent *RPS15a* genes and proteins.

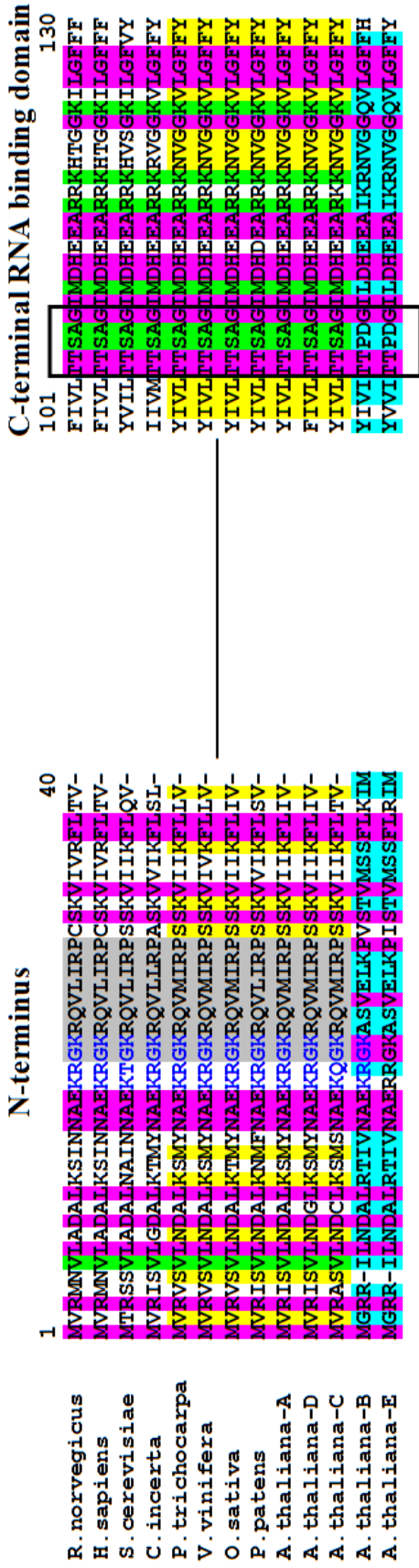


Figure 3.1. An alignment of the N-terminus and C-terminus of selected eukaryotic RPS15as. The deduced amino acids of the various RPS15as were aligned using ClustalW2 from the European Molecular Biology Laboratory-Bioinformatics Institute. Identical amino acids shared among: all eukaryotic orthologs are highlighted in magenta; all orthologs except type II RPS15aB and RPS15aE are highlighted in green; all plant RPS15a orthologs except RPS15aB and RPS15aE are highlighted in yellow, type II RPS15a isoforms are highlighted in blue. The experimentally determined nuclear localization signal of yeast is highlighted in grey. The putative nucleolar binding motif 'KRGK' is shown in blue at the N-terminus. The conserved (S/T)-T-(S/T/P)-X-G is boxed.

considered a signature motif in archaea (*Methanococcus jannaschii* RPS8), eukarya (Human and plant RPS15a, Figure 3.1) and bacteria (*Thermus thermophilus* RPS8). Specifically, cytosolic RPS15as contain a T-T-S-A-G motif, whereas the mitochondrial type II RPS15as contain the T-T-P-D-G motif (Figure 3.1), where the proline substitution, common in the genus *Methanococcus*, likely functions in stabilization. It has been suggested that this conserved “pentapeptide motif” is important for the recognition of a specific structural site on small subunit rRNAs (Tishchenko et al., 2001).

In an attempt to identify other type II RPS15a ortholog genes in other angiosperms, Arabidopsis type II RPS15a sequences were used to search against the National Center for Biotechnology Information EST database (using tBLASTn available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Four new angiosperm “unknown proteins” were found to be good candidates as type II RPS15as: *Populus triocarpa* (accession # EF146122.1), *Elaeis guineensis* (accession # EU284980) *Vitis vinifera* (accession # AM431407), and *Picea sitchensis* (accession # EF082927); proteins share 79%, 79%, 74%, and 63% putative polypeptide identity with RPS15aB and 81%, 79%, 79% and 66% amino acid identity with RPS15aE (Table 3.2).

3.2. mRFP-tagged RPS15aB and RPS15aE localize to the mitochondria

To determine if the differences within the N-terminal domains of type I and type II RPS15as affect localization of the evolutionary divergent proteins, RPS15aB and RPS15aE were fused to monomeric RFP (mRFP), linked by glutathione-s-transferase (GST) (total molecular weight ~ 72 kDa). This arrangement produced a fluorescent protein with a molecular weight significantly larger than the size exclusion limit of the nuclear pore which is 40-60 kDa. Therefore any localization patterns identified would be the result of active or facilitated transport. It has previously been demonstrated that fluorescent tags do not affect incorporation of r-proteins into ribosomes (Krüger et al., 2007). As RPS15aB and RPS15aE do not contain the S22-like NLS and have been shown to be imported into soybean mitochondria *in vitro* (Adams et al., 2002), I postulated that the type II RPS15as do not localize to the nucleolus, but do localize to the mitochondria. The mitochondrial marker, β -ATPase (the β subunit of ATPase) fused to a N-terminal GFP5 tag localizes to the inner

Putative plant type II RPS15as	Arabidopsis RPS15aB	Arabidopsis RPS15aE
<i>Medicago truncatula</i> RPS15a	80%	81%
<i>Populus Triocarpa</i> RPS15a	79%	81%
<i>Elaeis guineensis</i> RPS15a	79%	79%
<i>Vitis vinifera</i> RPS15a	74%	79%
<i>Zea mays</i> RPS15a	71%	79%
<i>Oryza sativa</i> RPS15a	74%	77%
<i>Solanum lycopersicum</i> RPS15a	75%	74%
<i>Picea sitchensis</i> RPS15a	63%	66%

Table 3.2. Polypeptide sequence identity shared among plant type II RPS15as. Bolded species indicates previously identified type II RPS15as (the legume, *Medicago truncatula* and tomato, *Solanum lycopersicum* type II RPS15as were identified in Adams et al. (2002), whereas Chang et al. (2005) identified type II RPS15as in maize, *Zea mays*, and rice *Oryza sativa*. New putative type II RPS15as identified in this thesis are non-bolded, and include cottonwood (*Populus Triocarpa*), African oil palm (*Elaeis guineensis*), grapevine (*Vitis vinifera*) and sitka spruce (*Picea sitchensis*). Amino acid identities shared between the plant type II RPS15as and Arabidopsis RPS15aB and -E are indicated.

membrane of mitochondria (Logan and Leaver, 2000). A nucleolar marker, AtFIBRILLARIN2 (FIB2) with a C-terminal enhanced GFP (EGFP) tag was also used in the experiments (Barneche et al., 2000). Free mRFP and EGFP localize to the cytoplasm and nucleoplasm of the epidermal cells (data not shown). FIB2 is a component of the nucleolus and plays crucial roles during rRNA processing events. FIB2, β -ATPase, and fluorescently tagged RPS15aB/-E were transiently expressed in tobacco leaf epidermal cells, via *Agrobacterium tumefaciens* (*A. tumefaciens*) mediated transformations, and visualized using confocal laser scanning microscopy (CLSM) (Figure 3.2 panels A-E). The localization pattern of RPS15aB and RPS15aE appeared non-uniform (Figure 3.2 C & D), and unlike the pattern observed for FIB2 (Figure 3.2 B). Co-expression of fluorescent RPS15aB and RPS15aE with the mitochondrial marker, GFP5- β -ATPase, or the nucleolar marker, FIB2-EGFP demonstrated clear overlapping localization with GFP5- β -ATPase (Figure 3.2 F & G) and no overlap with FIB2-EGFP (Figure 3.2 H & I).

These results indicate that unlike type I cytosolic RPS15as, which localize to the nucleolus (J. Hulm, Ph.D thesis), type II Arabidopsis RPS15aB and RPS15aE target to tobacco mitochondria, where they are likely incorporated into mitochondrial ribosomes. Preliminary proteomics data suggests that isolated mitochondrial ribosomal samples contain only type II RPS15a isoforms (Carroll et al., 2007).

3.3. RPS15a expression and purification

While the expression levels of histidine-tagged RPS15aA/F, -D, -E fusion proteins were very high in *Escherichia coli* (*E. coli*) Tuner cells, to optimize, a time course of expression was conducted. The optimal induction period was determined as 5 h, after which the expression for RPS15aA/F (Figure 3.3), RPS15aD, and RPS15aE was the strongest and contained the most soluble RPS15a. Cells induced with IPTG for 5 h were used for protein extraction and subsequent nickel-nitrilotriacetic acid (Ni-NTA) purification. Purification of histidine-tagged RPS15a was achieved by Ni-NTA affinity chromatography under non-denaturing conditions. All of the subsequent flow through, wash, and elution fractions were analyzed by SDS-PAGE analysis. The eluates were analyzed by western blots (Figure 3.3), with proteins visualized by a chemiluminescence imager, the EpiChem Darkroom, Bioimaging Systems. Western blots confirmed that the RPS15a proteins were successfully

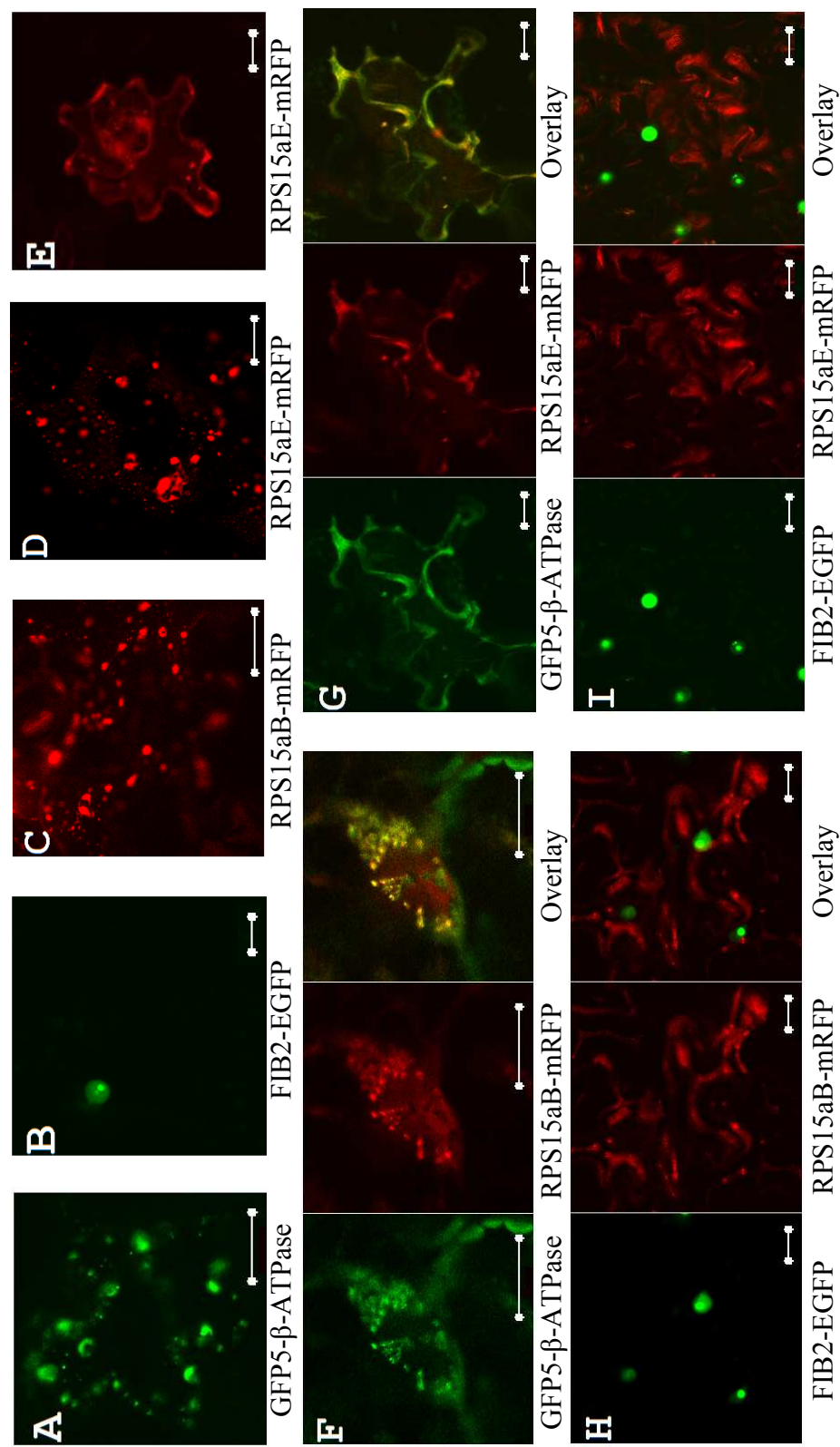


Figure 3.2. Mitochondrial localization of RPS15aB and RPS15aE. CLSM images of tobacco leaf epidermal cells transiently expressing GFP5- β -ATPase (A), FIB2-EGFP (B), RPS15aB-mRFP (C), RPS15aE-mRFP (D & E), and co-expressing FIB2-EGFP or GFP5- β -ATPase with RPS15aB-mRFP (F & H) and FIB2-EGFP or GFP5- β -ATPase with RPS15aE-mRFP (G & I). Scale bars = 20 μ m.

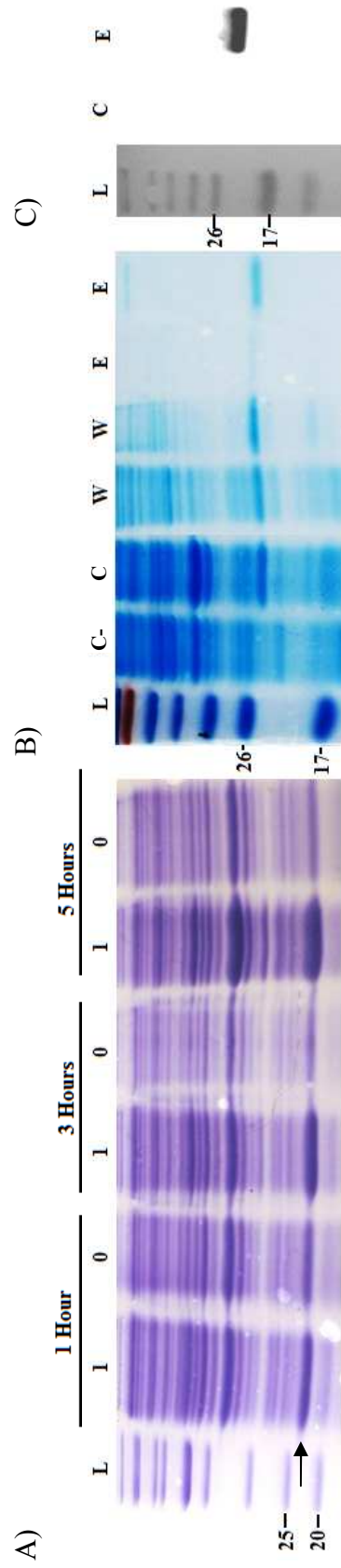


Figure 3.3. Protein expression and purification of histidine-tagged RPS15aA/F from *E. coli* Tuner cells. L: Molecular weight markers (-in kDa, Fermentas). A) Protein expression profile of total cell lysate in hours indicated after 1 mM (1) or 0 mM (0) IPTG induction. Arrow indicates the increasing expression of RPS15aA/F (~23 kDa on gel). B) Ni-NTA column fractions; C, crude fraction from 5 h IPTG induced cells, C-, non induced cells, W, wash fractions, E, elution of purified fusion protein. Proteins were visualized by Coomassie staining. C) Western analysis of 10 µg of the purified protein (from E) or the negative control (C, with no IPTG induction) using anti-6x-histidine tag antibody. Histidine-tagged RPS15a proteins were visualized using the enhanced chemiluminescence system, Bioimaging Systems.

purified; a band corresponding to the size of the predicted RPS15aA/F protein (~23 kDa) and in the appropriate lane (IPTG induced cells) was observed (Figure 3.3).

3.4. *In vitro* transcription

Complete digestion of pBSKS+ carrying the various 18S rDNA and RPS15a cDNA in fragments (sections 2.6) was verified on 1 % agarose gels (Figure 3.4 A). *In vitro* transcription from these templates resulted in at least 8 µg RNA from a 1 µg template in 30 minutes. All *in vitro* transcribed RNA products were heated at 70°C and separated on non denaturing agarose gels containing ethidium bromide (0.5 µg/mL) in order to assess length and quality of the RNA. After separation, agarose gels were visualized on a UV transilluminator and RNA bands were assessed. RNA transcripts of an appropriate size (Table 3.2) and integrity were observed (Figure 3.4 B). The various RNA molecules were considered pure as they were single non-degraded bands in each respective *in vitro* transcription reaction lane (Figure 3.4 B).

3.5. *RPS15aA/F and RPS15aD bind cytoplasmic 18S rRNA*

Although a nucleolar localization pattern of RPS15aA/F and RPS15aD had been previously established (J. Hulm, Ph.D thesis), these results gave no indication that either RPS15a isoforms interact with 18S rRNA in the small subunit. Electrophoretic shift assays (EMSAs) were employed to determine if either isoform, RPS15aA/F and/or RPS15aD, binds to the small subunit rRNA. *In vitro* synthesized cytoplasmic 18S rRNA was used in binding assays together with various concentrations of RPS15a to determine the binding characteristics for each isoform. Due to the large size of 18S rRNA, agarose gels were used as an alternative to traditional polyacrylamide gels. RNA-protein complex formation was determined by non-denaturing agarose gel electrophoresis. All r-protein-RNA complex shifted bands were observed relative to reactions containing no RPS15aA/F or RPS15aD and the negative control, BSA (Figure 3.5 A & B). BSA, a protein that does not bind nucleic acids (Lu et al., 2007), due to its negative charge, has been previously used as a negative control in r-protein-rRNA binding assays (Yeh and Lee, 1998) as well as DNA EMSAs (Wang et al., 2007). In addition, RPS15aA/F and RPS15aD were unable to interact

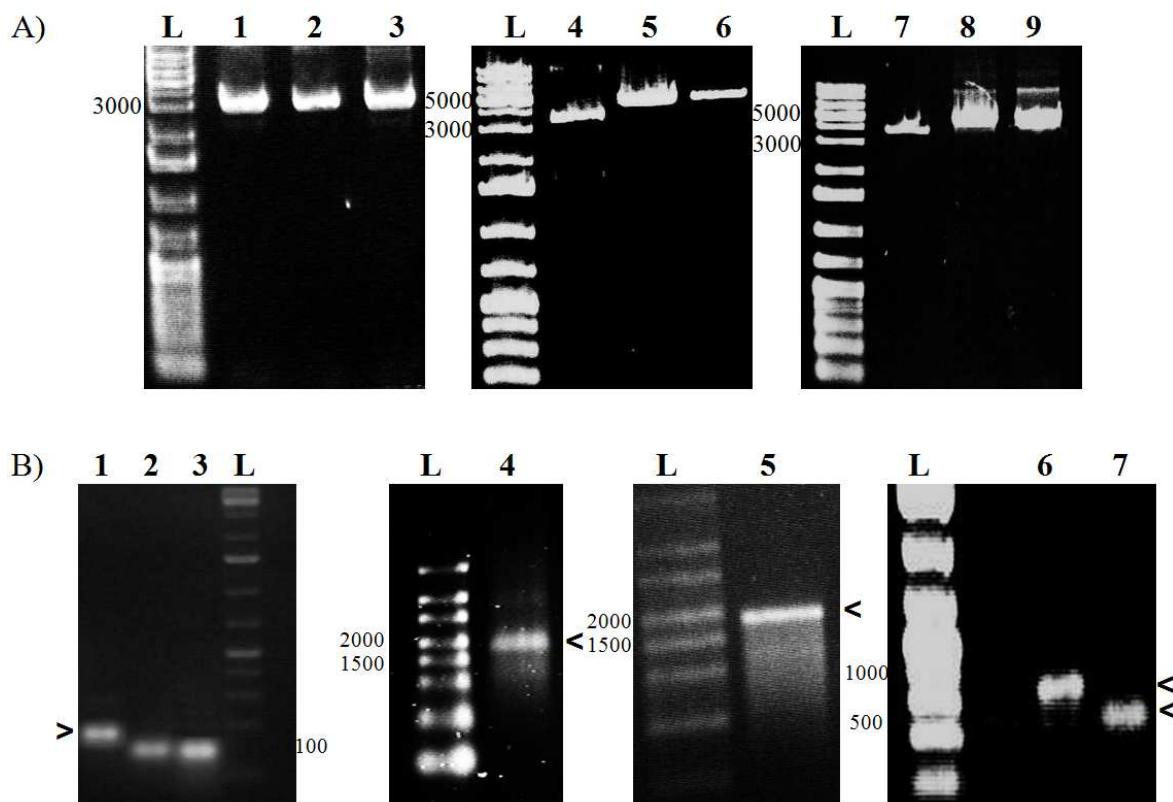


Figure 3.4. *In vitro* transcription templates and reaction products. A) Number of base pairs relative to the GeneRuler 1 kb DNA Ladder Plus (Fermentas) is indicated on the left hand side of the ladder. L=ladder (with value of marker indicated), 1: helix 23 loop 1-6; 2: helix 23 loop 1-2; 3: helix 23 loop 4-6; 4: RPS15aE cDNA 5-6: cytoplasmic 18S rDNA 7: RPS15aD cDNA; 8+9: mitochondrial 18S rDNA. B) Arrow heads indicate the various RNA molecules generated from *in vitro* transcription of the DNA templates in (A): 1: cytoplasmic helix 23 loop 1-6 ~213 nt; 2: cytoplasmic helix 23 loop 4-6 ~119 nt; 3: cytoplasmic helix 23 loop 1-2 ~94 nt; 4: mitochondrial 18S rRNA ~1900 nt; 5: cytoplasmic 18S rRNA ~1800 nt; 6: RPS15aD mRNA ~743 nt; 7: RPS15aE mRNA ~668 nt.

RNA obtained	Size in nt
cytoplasmic 18S rRNA	1808
mitochondrial 18S rRNA	1935
cytoplasmic helix 23 loop 1-6	213
cytoplasmic helix 23 loop 1-2	94
cytoplasmic helix 23 loop 4-6	119
RPS15aD mRNA	743
RPS15aE mRNA	668

Table 3.3. Predicted sizes (in nucleotides) of the RNA fragments obtained from *in vitro* transcription.

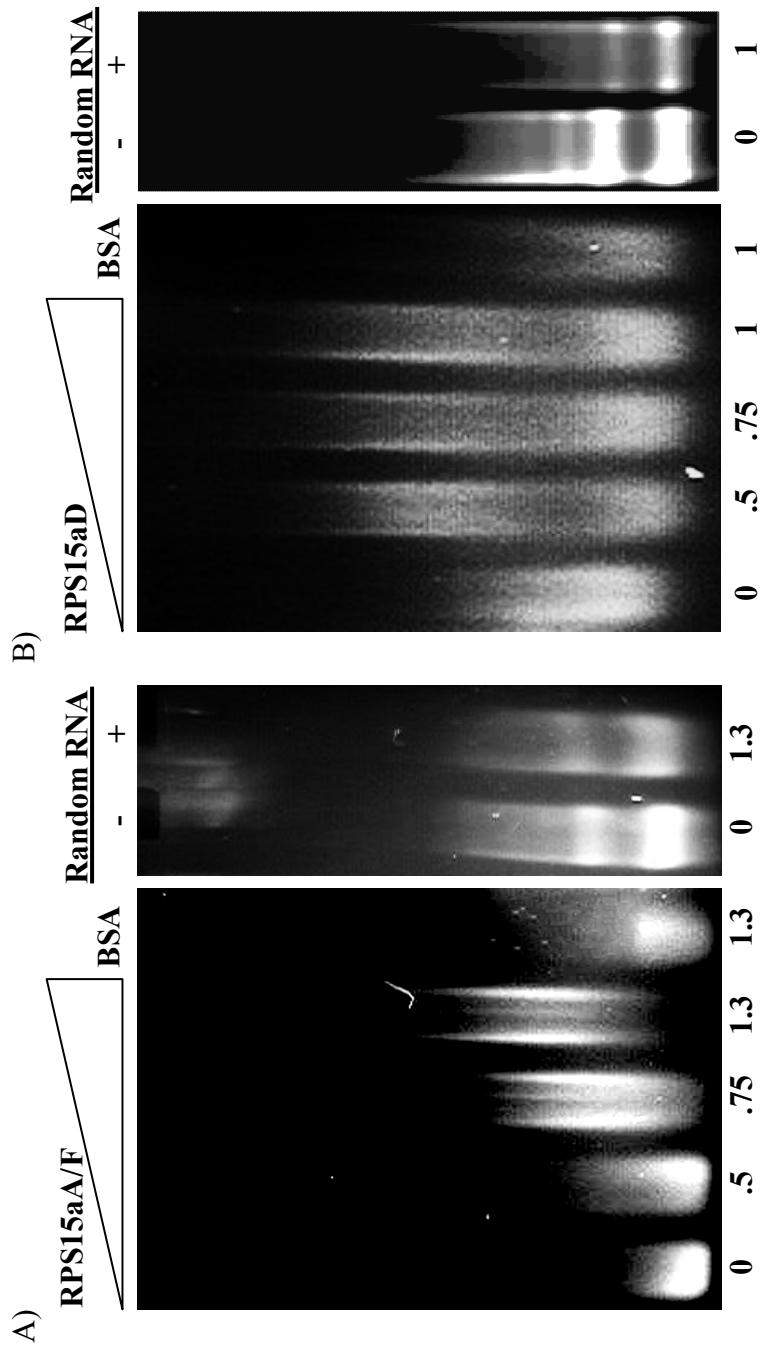


Figure 3.5. Detecting type I RPS15a-cytoplasmic 18S rRNA binding. A fixed concentration of 18S rRNA (100 ng) or the negative control (random RNA), was pre-incubated in binding buffer at 37°C followed by the addition of increasing amounts of RPS15aA/F (A) or RPS15aD (B) (indicated on the bottom of the figure in μg) and further incubation at 37°C for 15 min. Binding products (10 μL) were separated on 1% agarose gels followed by staining with SYBR Green. Gels were visualized with an Epichemi3 Darkroom.

with a random RNA molecule of the same size as 18S rRNA (Figure 3.5 A & B), suggesting that the cytosolic RPS15a isoforms, RPS15aA/F and RPS15aD, bind cytoplasmic 18S rRNA with some degree of specificity. The random RNA in its native form also appears as two bands, which represents different structures of the single RNA species. These results are consistent with a primary rRNA-binding role for RPS15a on cytoplasmic 18S rRNA. Each individual EMSA in this thesis is a representative of five independent *in vitro* transcription product replicates.

3.6. RPS15aE binds mitochondrial and cytoplasmic 18S rRNA

Although RPS15aE localizes to the mitochondria (section 3.2), whether the r-protein is being incorporated into mitochondrial ribosomes is still to be determined. *In vitro* synthesized mitochondrial and cytoplasmic 18S rRNA were used to determine the rRNA binding characteristics of RPS15aE, with RNA complex formation determined by non-denaturing agarose gel electrophoresis. Consistently, a shifted band corresponded to the lanes containing excess RPS15aE with both cytoplasmic 18S rRNA (Figure 3.6 A) and mitochondrial 18S rRNA (Figure 3.6 B). No shifted bands were observed in reactions lacking RPS15aE or with the BSA negative control. In addition, RPS15aE does not interact with a random RNA molecule of the same size as 18S rRNA. These results suggest that RPS15aE binds cytoplasmic 18S rRNA and mitochondrial 18S rRNA. The high degree of amino acid conservation at the C-terminus of all of the RPS15a isoforms, regardless of evolutionary divergence, implies that rRNA binding is conserved, and that the different RPS15a isoforms likely bind similar RNA targets. To determine if all of the isoforms are able to bind to both the cytoplasmic and mitochondrial 18S rRNA, further EMSAs were conducted replacing type II RPS15aE with type I RPS15aA/F and RPS15aD. Identical experimental conditions were used for binding reactions involving RPS15aA/F or RPS15aD with mitochondrial 18S rRNA. No detectable shifts were observed for type I RPS15aA/F (Figure 3.7 A) and RPS15aD (Figure 3.7 B) indicating that these two isoforms are unable to interact with mitochondrial 18S rRNA. These data suggest that only type II RPS15aE, and not type I RPS15aA/F or -D, interacts with mitochondrial 18S rRNA. However, in order to definitively report that RPS15aB and RPS15aE are incorporated into mitochondrial ribosomes, the ribosomes must be isolated, purified, and the protein components analyzed.

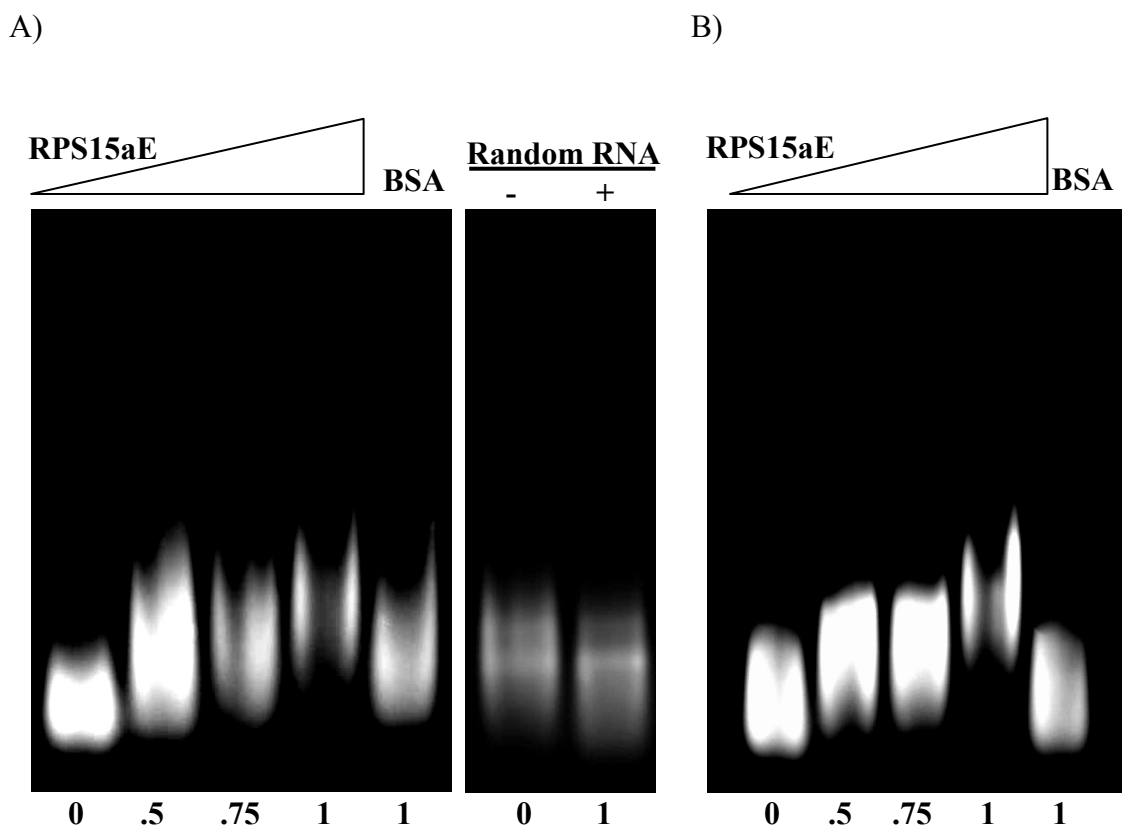


Figure 3.6. Electrophoretic shift assays detecting RPS15aE-18S rRNA interactions. Binding assays of increasing concentrations (indicated in μg at the bottom of the figure) of RPS15aE, or the negative control BSA, incubated with 100 ng of either cytoplasmic 18S rRNA (A) or mitochondrial 18S rRNA (B) or an RNA product of the same size as 18S rRNA (A) at 37°C for 15 min. Binding products (10 μL) were separated on 1% agarose gels followed by staining with SYBR Green and visualization with an Epichemi3 Darkroom.

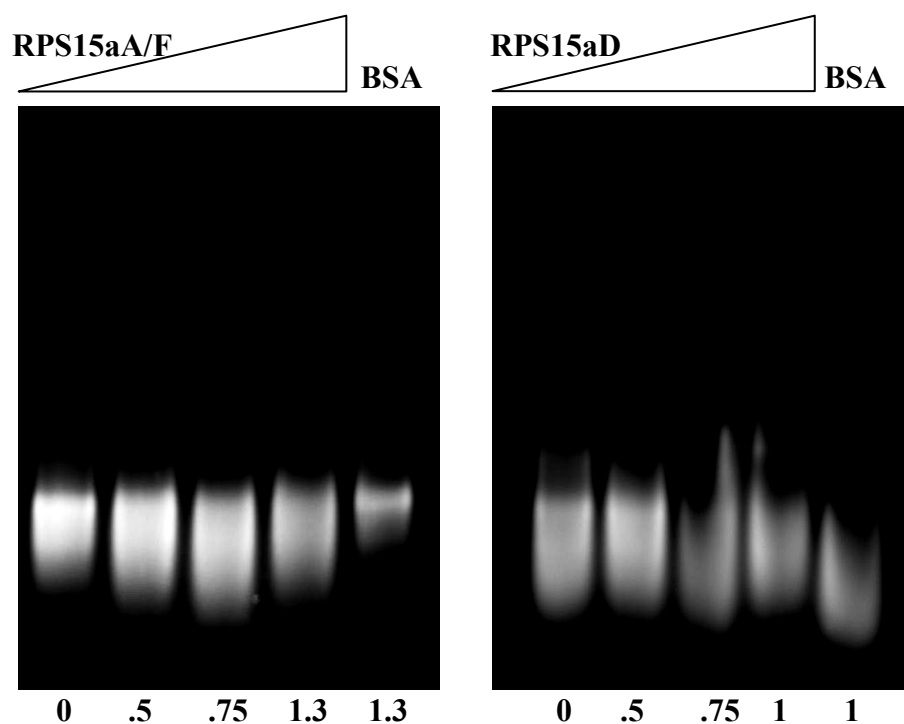


Figure 3.7. Electrophoretic shift assays detecting no interaction between RPS15aA/F, or -D with mitochondrial 18S rRNA. Binding assays of increasing amounts (indicated in µg at bottom of figure) of type I r-protein RPS15aA/F (A) or RPS15aD (B) incubated with a constant concentration (100 ng) of mitochondrial 18S rRNA at 37°C for 15 min. The 10 µL binding products were separated on a 1% agarose gel followed by staining with SYBR Green and visualized with an Epichemi3 Darkroom.

3.7. Different RPS15a isoforms preferentially bind different 18S rRNA loop fragments

To determine where RPS15aA/F and RPS15aD bind on cytoplasmic 18S rRNA (Figure 1.1), the *E. coli* RPS8 confirmed RNA binding site (helix 21) was aligned with the Arabidopsis 18S rRNA sequence and the corresponding region, helix 23 (or loop 1-6), was identified as the putative RPS15a rRNA binding site. Helix 23 loop 1-6 was further subdivided into two loop fragments, loop 1-2 and loop 4-6. In addition to binding to full length cytoplasmic 18S rRNA, RPS15aA/F interacted with one 18S rRNA helix 23 loop 4-6 fragment (Figure 3.8), whereas RPS15aD interacted with all of the helix 23 loop fragments (Figure 3.9 E). Consistently, RPS15aD interacted with each of the three loop fragments forming a complex as a function of increasing RPS15aD concentration. The specificity of these interactions was verified by the inability of BSA to interact with the rRNA loop fragments under the same experimental conditions. All r-protein-RNA complex shifts are relative to the lanes with no RPS15aD, and the negative control BSA. It appears that both of the cytosolic RPS15a isoforms bind helix 23 loop 4-6, whereas RPS15aD also binds to the entire helix 23 loop and its component fragments. In addition to binding to full length cytoplasmic and mitochondrial 18S rRNA, RPS15aE also interacted with all of the cytoplasmic 18S rRNA helix 23 loop fragments (Figure 3.9). Consistently, RPS15aE interacted with each of the three loop fragments forming a small complex, although the most significant shift was observed with binding to the entire helix 23 (Figure 3.9.B). The specificity of these interactions was verified by the inability of BSA (Figure 3.9) or denatured RPS15aE (data not shown) to interact with the RNA fragments under the same experimental conditions.

3.8. Supershift EMSAs confirm specificity of RPS15a-18S rRNA binding

Antibody supershift assays were conducted in order to further confirm the RPS15a-18S rRNA helix 23 loop 4-6 binding specificity. This experiment was conducted with two approaches; the anti-6x-histidine antibody was added prior to complex formation, or after complex formation. No supershifts were observed when the anti-6x-histidine antibody was incubated with the RPS15a proteins prior to the addition of RNA (data not shown). This suggests that antibody binding to the RPS15as resulted in an altered structure, or blocked the

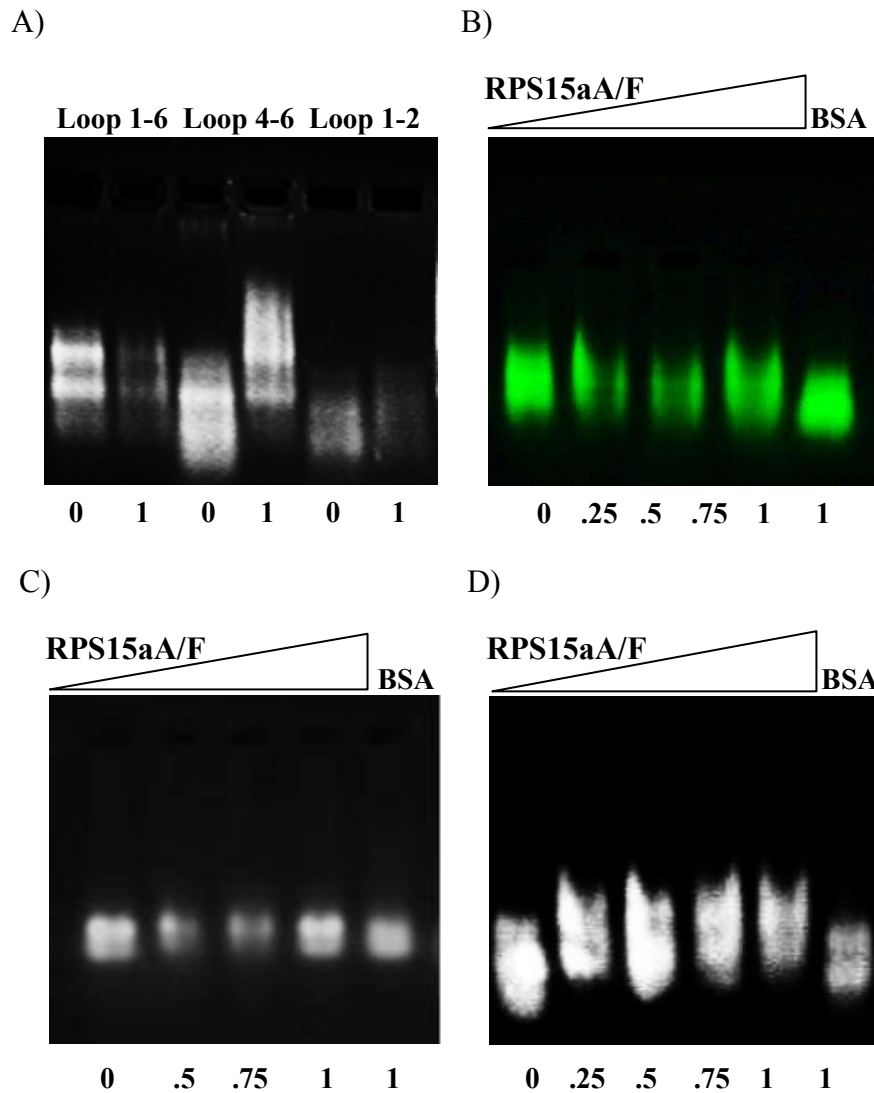
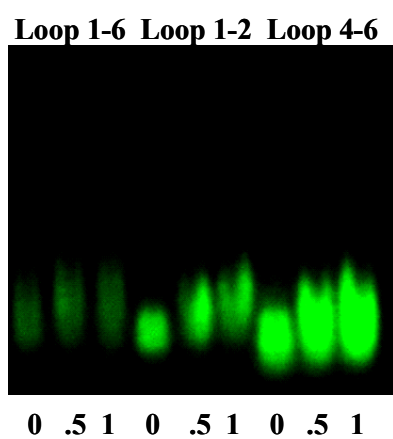


Figure 3.8. Electrophoretic shift assays detecting an interaction between RPS15aA/F and cytoplasmic 18S rRNA helix 23 loop 4-6. Electrophoretic shift assays for RPS15aA/F with 100 ng of all helix loop fragments of 18S rRNA (A), helix 23 loop fragment 1-6 (B), 1-2 (C), or 4-6 (D). RNA molecules were pre-incubated in binding buffer at 37°C followed by incubation with increasing amounts of RPS15a (indicated on the bottom of the figure in μg) and further incubated at 37 °C for 15 min. Reactions (10 μL) were separated on a 1% agarose gel followed by staining with SYBR Green.

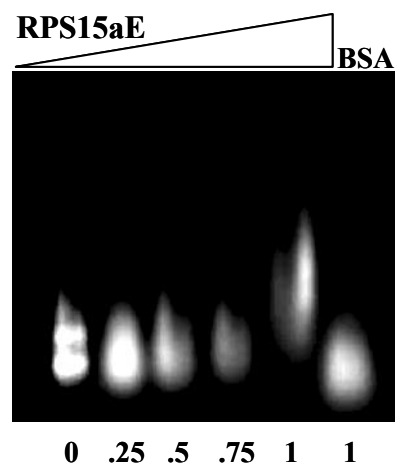
Figure 3.9. RPS15aD and RPS15aE bind to helix 23 of cytoplasmic 18S rRNA.

Representative electrophoretic shift assays for RPS15aD and RPS15aE with all helix loop fragments (A), the entire helix 23 (B), loop 1-2 of helix 23 (C), and loop 4-6 of helix 23 (D) are shown. A fixed concentration (100 ng) of 18S rRNA helix 23 loop fragment 1-6, 1-2, or 4-6 was pre-incubated in binding buffer at 37 °C followed by incubation with increasing amounts of RPS15aD or RPS15aE (indicated on the bottom of the figures in μg) and further incubated at 37°C for 15 min. Reactions (10 μL) were separated on a 1% agarose gel followed by staining with SYBR Green. Non-denaturing polyacrylamide gels (E & F) were also employed to confirm some of the interactions observed on agarose gels. The 18S rRNA loop fragment (100 ng) involved in the binding reactions is indicated on the top of the figure as well as the concentration (μg) of RPS15aD (E) or RPS15aE (F) indicated on the bottom of the figure.

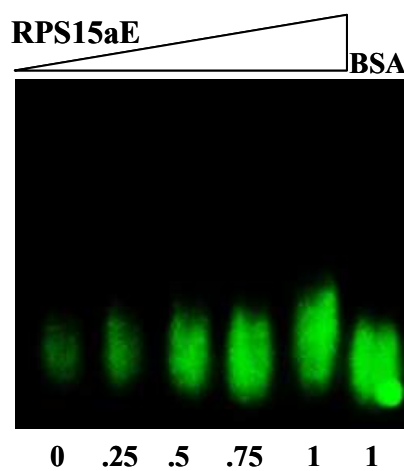
A)



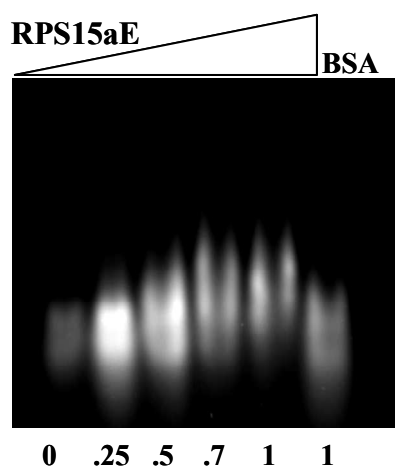
B)



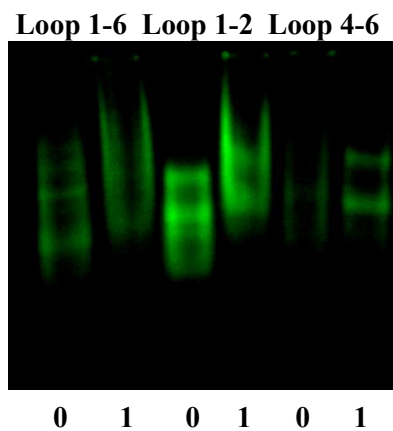
C)



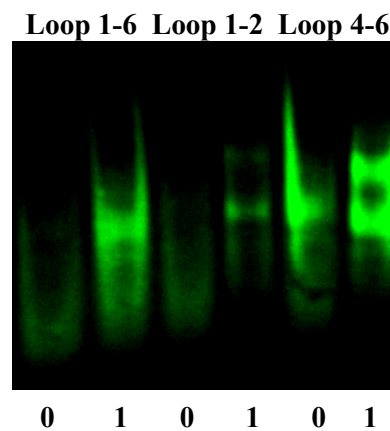
D)



E)



F)



binding domain, thus abolishing r-protein-RNA complex formation. For this reason, the anti-6x-histidine antibody was added to the incubation mix after the initial RPS15a-rRNA incubation. It was determined that 0.5 µg of anti-6x-histidine antibody was optimal for the formation of a tertiary antibody-RPS15a-RNA complex, producing a large shift (Figure 3.10). Similar results have been previously reported in supershift assays, where a high antibody concentration, in this experiment 1 µg of anti-6x-histidine antibody, either obliterated or reduced a shift or produced no greater shift (Pirkkala and Sistonen, 1999). The lanes containing binding reactions and 0.5 µg anti-6x-histidine probe contained a slowly migrating “supershifted” band (Figure 3.10 A & B), as well, supershifts were observed for binding reactions with 0.25 µg anti-6x-histidine antibody (Figure 3.10 C). No supershifted band was observed when 0.25 µg (data not shown), 0.5 µg (data not shown) or 1 µg of the control polyclonal antibody, anti-RFP, was added to the binding mixture. In addition, the anti-6x-histidine antibody did not interact with anything in the BSA mix. These experiments confirm my earlier results (section 3.8 D & 3.9 D) that the shifts resulting from the addition of either RPS15aA/F or RPS15aE to 18S rRNA helix 23 loop 4-6 are due to specific protein-rRNA interactions.

In addition to helix 23 supershift assays, a RPS15aE-mitochondrial 18S rRNA supershift assay was also conducted (Figure 3.11). The resulting supershift suggests that RPS15aE also binds specifically to mitochondrial 18S rRNA (Figure 3.11). Due to limited quantity of RPS15aE, only 0.75 µg of the r-protein was used in the binding assays, although even with a reduced amount of RPS15aE present in the binding reactions, the anti-6x-histidine probe was able to detect the RPS15aE-mitochondrial 18S rRNA, yielding a large “supershifted” band (Figure 3.11). The supershift lane contained no free 18S rRNA or RPS15aE-18S rRNA complexes, suggesting that all of the 18S rRNA was bound to RPS15aE and subsequently to the antibody. This latter supershift assay needs to be repeated to confirm the results obtained in this study.

3.9. RPS15a binding to 18S rRNA is not blocked by RNA competition

To further examine the specificity of binding between RPS15as and cytoplasmic 18S rRNA helix 23 loop fragments, RNA competition binding experiments were carried out.

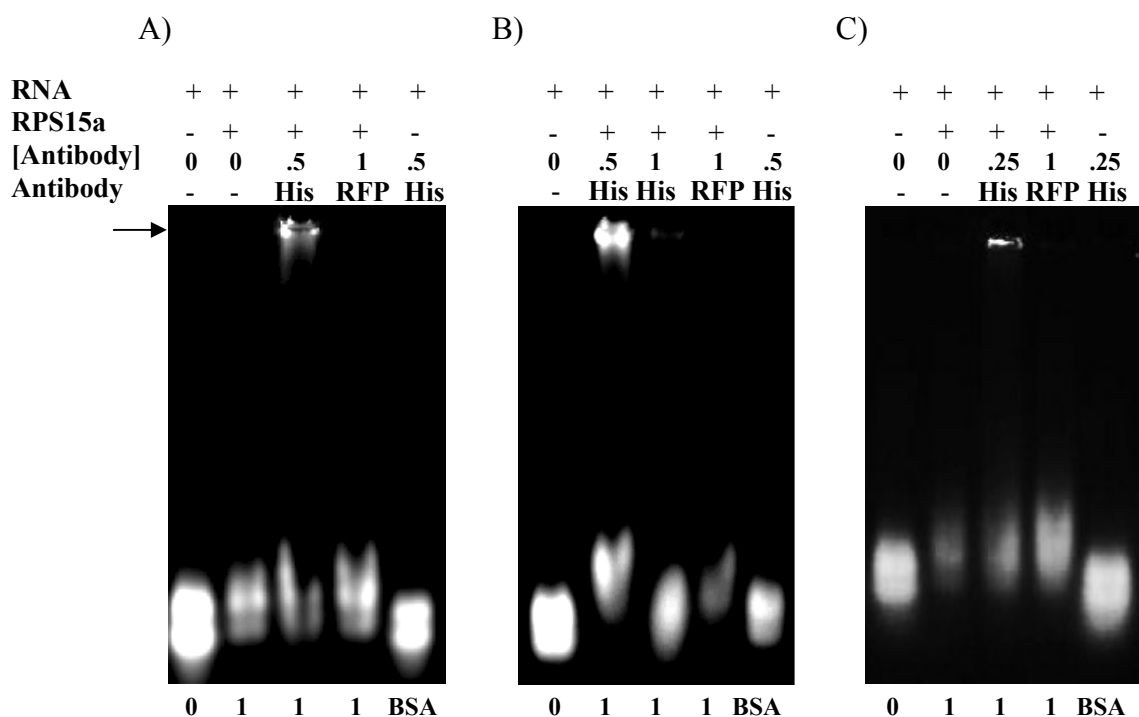


Figure 3.10. Detection of 18S rRNA loop helix 23-RPS15a complexes in EMSA supershift experiments with an anti-6x-histidine antibody. Amount of RPS15a in binding reactions indicated on the bottom of the figures. Supershifts, indicated on gels with black arrowhead, resulted from anti-6x-histidine [His] or anti-RFP [RFP] antibodies. The [His] antibody supershifted complexed RPS15aA/F-loop 4-6 (A & C) and RPS15aE-loop 4-6 (B), however the anti 6x-histidine probe did not produce a supershift for BSA-loop 4-6 reactions. In addition, no supershifts were observed with anti-RFP. Concentration (in μg) of the anti-6x-histidine antibody is also indicated.

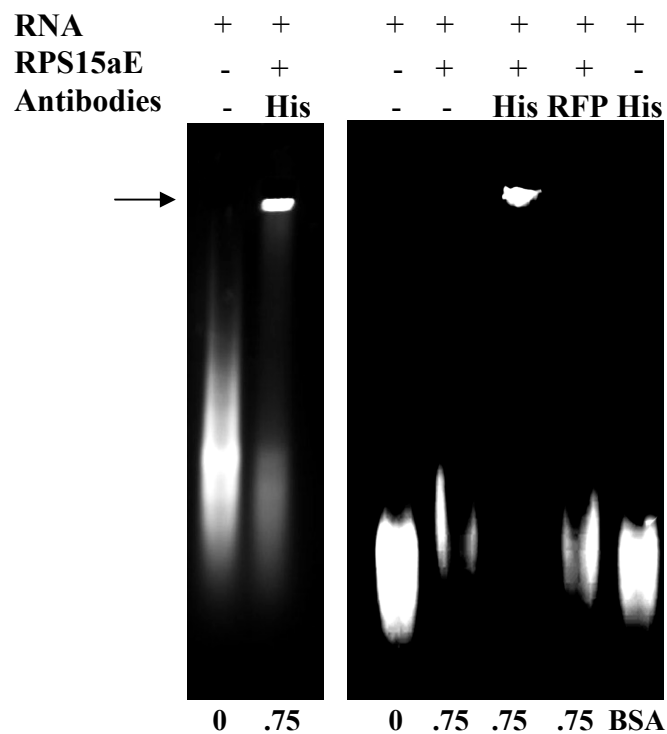


Figure 3.11. Detection of mitochondrial 18S rRNA-RPS15aE complexes in EMSA supershift experiments with an anti-6x-histidine antibody. Amount of RPS15a in binding reactions indicated on the bottom of the figures. Supershifts (arrow), used either anti-6x-histidine or anti-RFP antibodies. The anti- 6x-histidine antibody produced a supershift with the RPS15aE-mitochondrial 18S rRNA complex, and no shift with the BSA-mitochondrial 18S rRNA reaction. No supershifts were observed with the anti RFP antibody.

RPS15aD or RPS15aE mRNA and the cytoplasmic 18S rRNA helix 23 loop fragments, mixed in ratios of 5:1, 10:1, and 15:1, were added to 1 µg of RPS15aA/F,-D or RPS15aE. Typical results of competition binding experiments between cytoplasmic 18S rRNA helix 23 loop 4-6 and RPS15aD mRNA with RPS15aA/F are shown in Figure 3.12. RPS15aA/F interacted with only 18S rRNA helix 23 loop fragment 4-6 even in the presence of a 10 fold excess of RPS15aD mRNA under the present experimental conditions. Although RPS15aD mRNA contains elements of the 18S rRNA loop fragment, it failed to compete for RPS15aA/F binding. It is important to note that RPS15aD mRNA and RPS15a bound helix 23 loop 4-6 bands run at the same size under native agarose conditions. Similar results were obtained with RPS15aE and RPS15aE mRNA, although, at high concentrations (5-10 fold excess) RPS15aE mRNA was able to compete with cytoplasmic 18S rRNA loop fragment 1-6 and 1-2 for RPS15aE binding (Figure 3.13 A, B). Interestingly, RPS15aE mRNA failed to compete with cytoplasmic 18S rRNA helix 23 loop 4-6 for RPS15aE binding, suggesting that RPS15aE binding to this loop fragment is very specific (Figure 3.13). However, RPS15aE is mitochondrial, and therefore RPS15aE binding to 18S rRNA may not be as strong as type I RPS15a binding to 18S rRNA. These data suggest that the binding of RPS15a isoforms to 18S rRNA helix 23 is specific and that loop 4-6 is very important in this interaction.

3.10. RPS15as do not bind RPS15a mRNA

RPS8 binds its polycistronic mRNA in order to regulate the transcription of the *spc* operon containing the ORFs for eleven r-proteins, including its own ORF. To determine if this manner of regulation has been conserved, binding assays with RPS15aA/F and RPS15aD and their monocistronic transcripts were conducted. However, RPS15aF full length cDNA was unable to be cloned, and therefore RPS15aF mRNA was not obtained. Sequence alignments of full length 18S rRNA with RPS15aD mRNA (Figure 3.14 A) and RPS15aE mRNA (Figure 3.14 B) demonstrated 33% and 31% nucleic acid identity within the region highlighted, respectively. Consistently, gel mobility shift assays did not detect any interaction between an increasing amount of RPS15aA/F and RPS15aD and the RPS15aD transcript (data not shown) or between RPS15aE and its mRNA transcript (Figure 3.14 C).

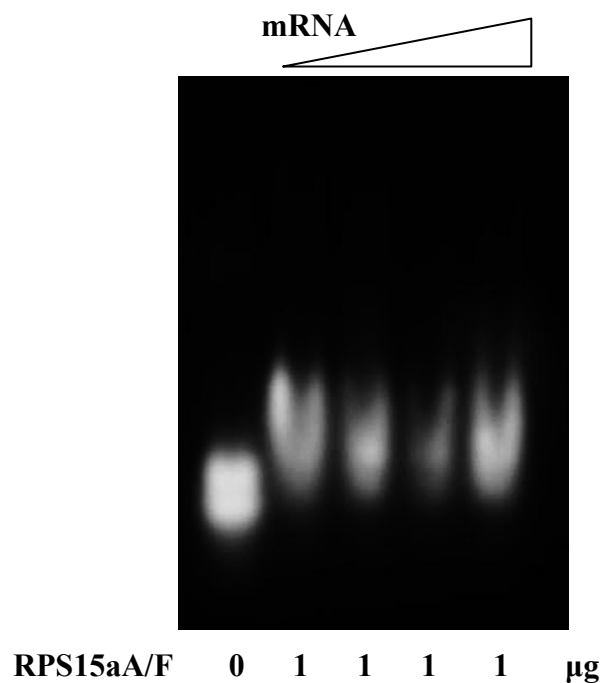


Figure 3.12. Competition between RPS15aD mRNA and cytoplasmic 18S rRNA helix 23 loop 4-6 for RPS15aA/F binding. A fixed concentration (100 ng) of 18S rRNA helix 23 loop 4-6 was mixed with increasing amounts (0, 0.5, 1, and 1.5 μg , lanes 2-5, respectively) of RPS15aD mRNA prior to adding RPS15aA/F (concentration indicated on bottom of figure in μg) to the binding reaction mixture. Reaction mixtures were separated through 1% agarose gels, and RNA-protein complexes detected by fluorescence imaging.

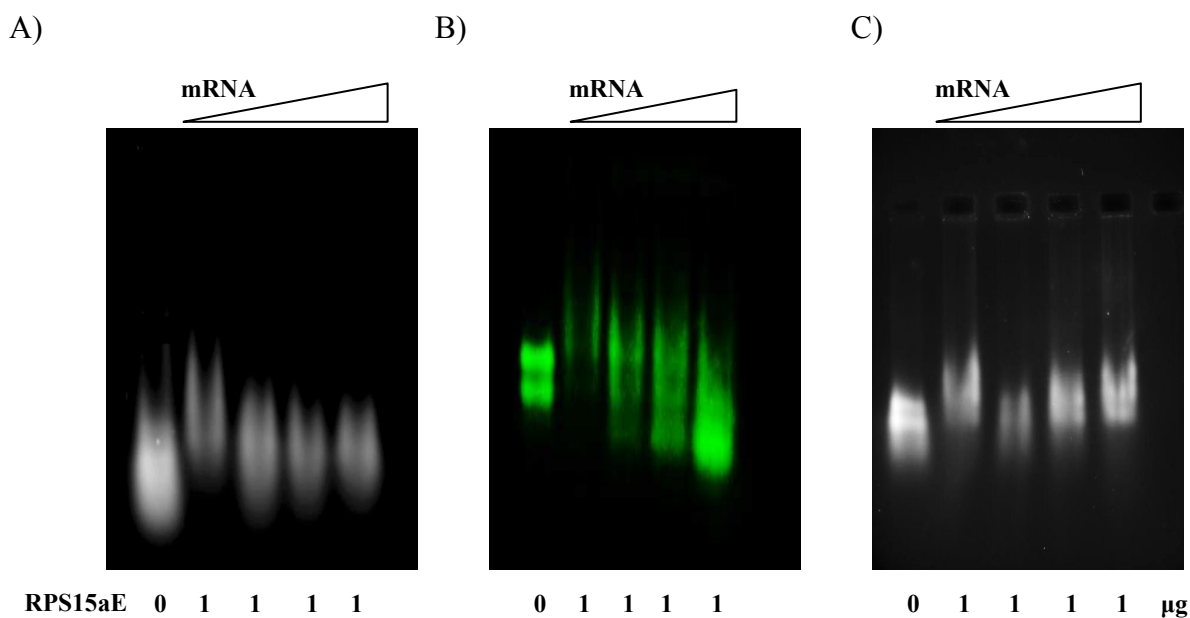


Figure 3.13. Competition between RPS15aE mRNA and cytoplasmic 18S rRNA helix 23 loop 1-6 (A), 1-2 (B) and 4-6 (C) molecules for RPS15aE binding. 18S rRNA loop fragments (100 ng) were mixed with increasing amounts (0, 0.5, 1, and 1.5 μg , lanes 2-5, respectively) of RPS15aE mRNA prior to the addition of RPS15aE (concentration indicated on the bottom of figures in μg). Reaction mixtures were separated by agarose gel electrophoresis. RNA-protein complexes were detected by fluorescence imaging.

A)

helix 21	-----GGGCGGTTTGTAAAGTCAGATGTGAAATCCC CGGGCTCA	501
D-cDNA	GGAAAGAGGCAGGTTATGATCAGGCCTTCTTCTAAAGTTATCATCAAGTTCCTTATCGTC	240
helix 23	CGCCTTTGGTGTGCATTGGTCGGCTTGCTCCCTTCGGTCGGCGAT-ACGCTCCTGGTCTTA	721

helix 21	A----CCTGGAACCTGATCTGATACTGGCAAGTT-GAG-----	556
D-cDNA	A----TGCAAAAGCACGGTT-ACATTGGCGAGTTTGAGTATGTTGATGACCACAGGTCTG	295
helix 23	ATTGGCCGGGTCGTGCTCCGGCGCTGTTACTTTGAAGAAATTAGAGTGCTCAAAGCAAG	781

B)

helix 21	-----GGGCGGTTTGTAAAGTCAGATGTGAA---ATCCCGGGCTCAA-----CCTGG	507
E-cDNA	CTTTTACTGAATAGCTTGAGCTAGCACATGGCAAGAACCCTTCTAGTGTCAAAGGATTCAA	480
helix 23	TGTGCATTGGTCGGCTTGCTCCCTTCGGTCGGCGATACGCTCTGGTCTTAATTGGCCGGG	731

helix 21	AACTGCATCTGATACTGGCAAGTTGGAG-----	556
E-cDNA	CAAAAGATTGAAGTTTCGGTCTTCTCCTGTTGCTTTTGTGTTTTTGTAACTTGCATTGT	540
helix 23	TCGTGCCTCCGGCGCTGTTACTTTGAAGAAATTAGAGTGCTCAAAGCAAGCCTACGCTCT	791

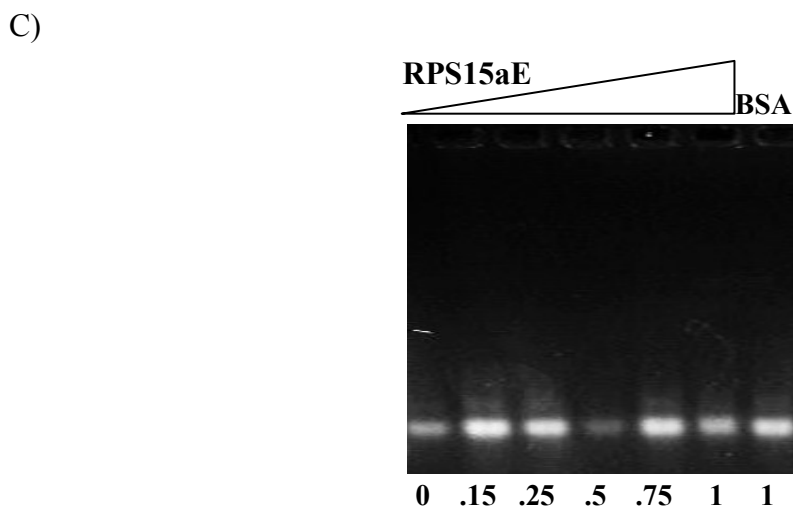


Figure 3.14. RPS15as do not bind their mRNA transcripts. A) Sequence alignment of *E. coli* 16S rRNA helix 21, Arabidopsis 18S rRNA helix 23 and Arabidopsis RPS15aD or RPS15aE full length cDNAs. All conserved nucleotides are indicated in green, whereas nucleotides shared between *E. coli* helix 21 and RPS15a full length cDNA [RPS15aD (A) or RPS15aE (B)] are indicated in blue, and conserved nucleotides shared only between RPS15a full length cDNA and *A. thaliana* 18S rRNA helix 23 are shown in magenta. C) Representative EMSA for RPS15a-RPS15a mRNA binding reactions. Increasing RPS15aE protein concentration (μ g) is indicated on the bottom of the figure. All reactions contain 100 ng of RPS15aE mRNA.

3.11. RPS15a does not bind 18S rDNA

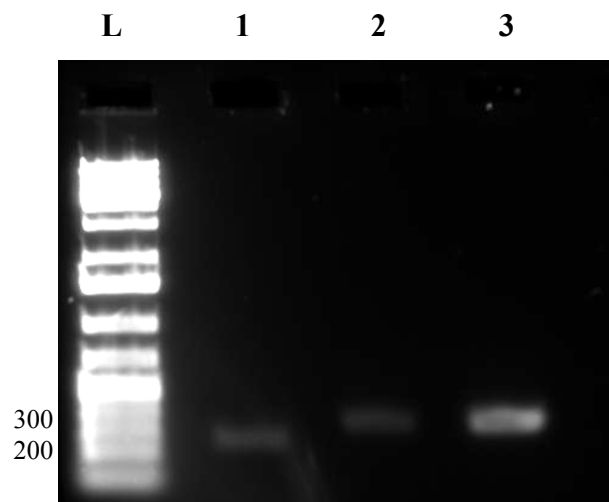
To investigate whether RPS15a could bind its complementary rDNA sequence during transcription, ribosomal single stranded DNA (ssDNA) and double stranded (dsDNA) sequences of helix 23 were used in EMSAs. 18S ribosomal dsDNA was obtained via PCR and its ssDNA moieties were obtained via linear PCR (Figure 3.15 A). In order to be confident that ssDNA was isolated, two different nucleic acid stains (ethidium bromide and SYBR Green II) with different affinities for dsDNA and ssDNA were employed. Ethidium bromide is considered the least sensitive ssDNA stain (Stothard et al., 1997), and so this stain was used to differentiate between dsDNA and ssDNA, whereas the sensitivities for SYBR Green II are equivalent for both dsDNA and ssDNA DNA (Stothard et al., 1997). Fifty ng of dsDNA and 50 ng of each template and non template ssDNA were separated on two non denaturing polyacrylamide gels followed by staining with either ethidium bromide and SYBR Green II. The dsDNA products were easily visualized following both ethidium bromide (data not shown) and SYBR Green II staining, whereas ssDNA products were readily visualized on the gel stained with SYBR Green II, yet barely visible on the polyacrylamide gels stained with ethidium bromide (data not shown). In this experiment, only cytosolic RPS15aA/F was tested, as RPS15aE is not present in the nucleolus (section 3.2) and therefore would not be present during rDNA transcription, and, in addition to BSA, which is used as negative control in ssDNA binding EMSAs (Lu et al., 2007), was used as a second negative control. As expected, no shifted bands were observed when RPS15aE was added to ribosomal dsDNA or either template/non template ssDNA (data not shown). Also, no shifts were observed when RPS15aA/F was incubated with helix 23 ribosomal dsDNA (Figure 3.15 B), non-template ssDNA (Figure 3.15 C), or template ssDNA (Figure 3.15 D). It is interesting that non-template ribosomal ssDNA contains identical sequence (thymine rather than uracil) to helix 23 rRNA to which RPS15aA/F binds, yet no shifts were detected.

3.12. Conservation of the putative 16S/18S rRNA binding sites for RPS8/S15a

RPS8 binds helix 21 of 16S rRNA which consists of nucleotides G588-G604 and C634-C651. It has been suggested that RPS8 recognizes 9 core elements within helix 21, residues 595-598 and 640-644, which undergo minor conformational changes upon RPS8

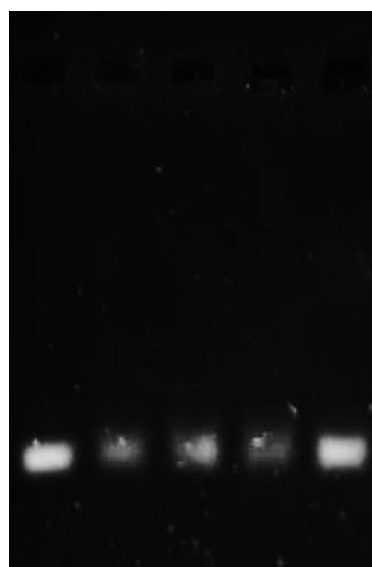
Figure 3.15. RPS15a does not bind 18S rDNA. A) Arabidopsis 18S ribosomal dsDNA (lane 1), template (lane 2) and non template ssDNA (lane 3). 18S ribosomal dsDNA (B), non-template ssDNA (C), or template ssDNA (D) was incubated with increasing amounts of RPS15aA/F (indicated in μg). Binding products (10 μL) were separated on a 1% agarose gel, stained with SYBR Green II and visualized with an Epichemi3 Darkroom.

A)



B)

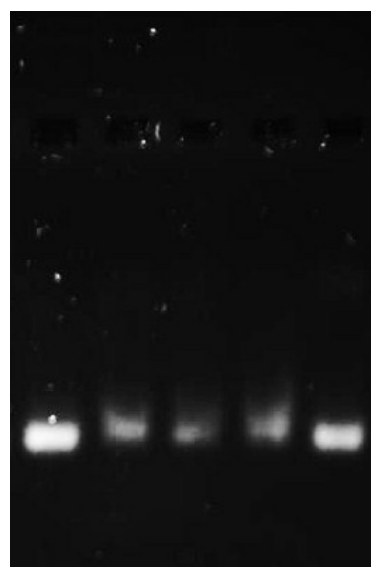
RPS15aA/F BSA



0 .5 .75 1.3 1.3

C)

RPS15aA/F BSA



0 .5 .75 1.3 1.3

D)

RPS15aA/F BSA

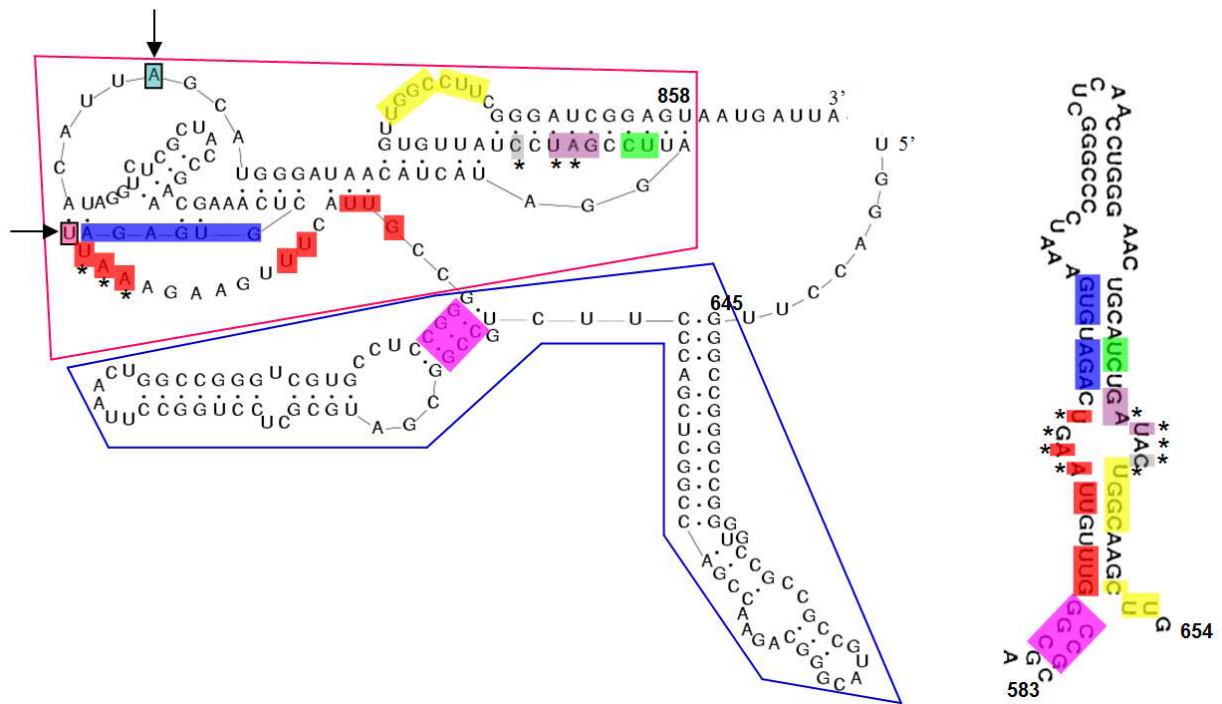


0 .5 .75 1.3 1.3

binding (Kalurachchi et al., 1997). By aligning the nucleotide sequence for *E. coli* 16S rDNA helix 21 with Arabidopsis 18S rDNA, the putative RPS15a RNA binding site can be highlighted. Based on predicted secondary structures of plant 18S rRNA, helix 23 is located within the same region as helix 21, in the central domain of the small subunit RNA. Although structurally the helices are quite different, the majority of the specific RPS8 binding site is conserved (Figure 3.16). Arabidopsis 18S rRNA helix 23 and *E. coli* 16S rRNA helix 21 are not only conserved at the nucleic acid sequence level, but they also contain a similar number of rRNA modifications. According to the mapped rRNA modifications database (Piekna-Przybylska et al., 2007), there is one pseudouridine and one methylation within helix 23 (loop 4-6 only) of Arabidopsis 18S rRNA: U763 (process likely guided by SnoR91) and A801 (methylation at this position likely guided by SnoR53Y) (Figure 3.16). It appears the pseudouridine modification is within proximity to the consensus “conserved core” region of helix 23. With only two of the total of 37 rRNA modifications in 18S rRNA, it appears that helix 23 is the least modified helix relative to the other 18S rRNA helices. This is comparable to helix 21 of the *E. coli* 16S rRNA, where none of the 11 base modifications are found in helix 21. This suggests that rRNA modifications are not important for RPS8 or RPS15a recognition/binding.

3.13. Identification of the putative mitochondrial 18S rRNA binding site for RPS15aE

RPS8 binds extensively to helix 21 (particularly to bulged bases 641-642 of the core domain) of 16S rRNA (Brodersen et al., 2002). The RPS8 binding site on helix 21 has been previously aligned to cytoplasmic 18S rRNA to identify the putative cytosolic RPS15a binding site (section 3.10). This binding site was further aligned with mitochondria 18S rRNA to identify the putative RPS15aE binding site. Overall, Arabidopsis mitochondrial 18S rRNA and *E. coli* 16S rRNA share 54.5% nucleic acid identity, with the highest percent sequence identity between helix 21 of *E. coli* 16S rRNA and Arabidopsis mitochondrial 18S rRNA found at the base of the loop structure (Figure 3.17). Helix 21 of Arabidopsis mitochondrial 18S rRNA was modeled after maize mitochondrial 18S rRNA using Sfold provided at the Wadsworth Bioinformatics Center (<http://sfold.wadsworth.org/>). Based on the conservation in sequence and possible structure of both helix 21s' (47% identity), one can



A. thaliana 18S rRNA Helix 23

E. coli 16S rRNA Helix 21

Figure 3.16. The probable RPS15a binding site within helix 23 of 18S rRNA. A) Predicted secondary structure of helix 23 of cytoplasmic 18S rRNA of *A. thaliana* (adapted from *Z. mays* SSU rRNA secondary structure model, Van de Peer et al., 2000) and B) the confirmed secondary structure of helix 21 of *E. coli* 16S rRNA (adapted from Kalurachchi et al., 1997). Conserved RPS8 bindings sites are shown in various colors highlighting corresponding regions with the conserved core indicated with asterisks. The putative 18S rRNA helix 23 binding site of RPS15a, split into loops 1-2 (outlined in blue) and 4-6 (outlined in pink), was identified based on sequence identity to *E. coli* helix 21. Modified rRNA of helix 23 loop 4-6 is indicated with an arrow and highlighted: U763 boxed and highlighted in pink; A801, boxed and highlighted in light blue. Helix 21 of 16S rRNA does not contain any modified bases.

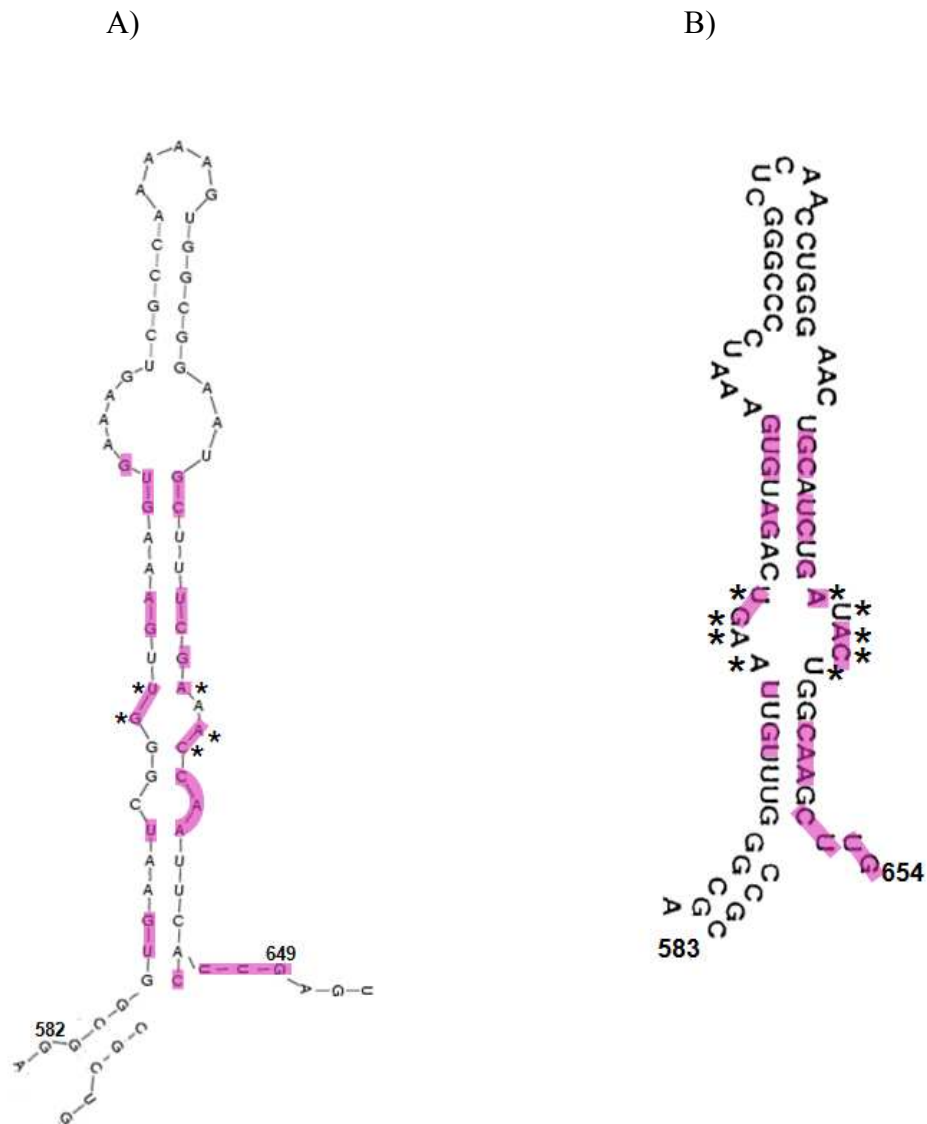


Figure 3.17. Probable mitochondrial 18S rRNA binding site for RPS15aE. Predicted secondary structure of A) helix 21 from Arabidopsis mitochondrial 18S rRNA (adapted from *Zea mays*, Chao et al., 1984 and predicted from Sfold, Wadsworth Bioinformatics Center). The predicted secondary structures of plant small subunit rRNAs are based on experimentally determined prokaryotic 16S rRNA structures. B) The confirmed secondary structure of helix 21 from *E. coli* 16S rRNA (adapted from Kalurachchi et al., 1997). The *E. coli* 16S rRNA helix 21 core domain, nucleotides 595-598 and 640-644, are highlighted with asterisks. Conserved RPS8 binding/recognition site nucleotides are highlighted in pink.

suggest that helix 21 of mitochondrial 18S rRNA is a strong candidate for RPS15aE binding. While the overall sequence similarity between Arabidopsis cytoplasmic 18S rRNA and Arabidopsis mitochondrial 18S rRNA is 43% with cytoplasmic 18S rRNA helix 23 and mitochondrial 18S rRNA helix 21 sharing 42% sequence identity, the predicted helix structures are extremely different in shape (Figure 3.16 & 3.17 A). However, the core RPS8 binding site appears to be conserved in Arabidopsis 18S rRNAs, particularly the cytoplasmic 18S rRNA helix 23, where 6 of the 9 core nucleotides are conserved (Figure 3.16). This is comparable to helix 21 of mitochondrial 18S rRNA where 5 of the 9 core nucleotides are conserved. Conservation of specific nucleotides and the predicted bulged structure in which they are located, suggests that as for RPS8 binding, these nucleotides are important in both type I and type II RPS15a site recognition and perhaps binding.

CHAPTER 4. DISCUSSION

4.1. Type I versus type II RPS15as

Members of the S8p family are among the few r-proteins that are found in all organisms and ribosome types (i.e. cytoplasmic, mitochondrial, plastidic; Lecompte et al., 2002) and are important in initial ribosome subunit assembly, as determined in prokaryotes. All members of the S8p family are conserved at the putative polypeptide sequence level. The amino acid sequence identity for prokaryotic *Methanococcus jannaschii* RPS8 shared with type I Arabidopsis RPS15a (41-45%) orthologs is comparable to the amino acid identity shared between the Arabidopsis type I and type II clades (49-52%). Although the type I and type II Arabidopsis RPS15a isoforms show differences at the amino acid sequence, relative to various prokaryotic RPS8s, the shared amino acid identities are equally different. For example, both Arabidopsis RPS15a clades share between 19-25% amino acid identity with *Thermus thermophilus* RPS8 and 20% amino acid identity with *E. coli* RPS8. The level of sequence identity between the Arabidopsis RPS15as and prokaryotic RPS8 is reflective of the level of sequence identity between rat or human RPS15a and the same prokaryotic species, highlighting the conservation in amino acid sequences between eukaryotic RPS15a orthologs.

Although cytosolic type I RPS15as are highly conserved between plants and animals, conservation of type II RPS15as has yet to be thoroughly analyzed. To date, no angiosperms contain a copy of RPS8 in their nuclear or mitochondrial genomes, only in the genomes of their chloroplasts. Adams et al. (2002) hypothesized that type II RPS15as accommodate this loss. Type II RPS15as have been identified in rice, maize, (*Osa S15a2* and *Zma S15a2*; Chang et al., 2005) tomato and legumes (which share ~75-80% amino acid identity with Arabidopsis type II RPS15as). The four new angiosperm unknown proteins in *Populus triocarpa*, *Elaeis guineensis*, *Vitis vinifera*, and *Picea sitchensis* (which share 79%, 79%, 74%, and 63% putative polypeptide identity with RPS15aB and 81%, 79%, 79% and 66% amino acid identity with RPS15aE, Table 3.2) all share extremely high sequence similarity

with type II RPS15as. This level of sequence similarity is much higher than that between Arabidopsis type I and type II RPS15as (Table 3.1) and as such, they are likely mitochondrial proteins. As more plant genomes are completely sequenced, it is likely that type II RPS15as will be identified in these genomes.

4.2. Mitochondrial localization of RPS15aB and RPS15aE

Consistent with the ability of RPS15aE to bind mitochondrial 18S rRNA, C-terminally tagged RPS15aB and RPS15aE fusion proteins were targeted to mitochondria of tobacco leaf epidermal cells (Figure 3.2). Combined, these results confirm a mitochondrial location for RPS15aB and -E. To date, there is little to no information on the mitochondrial localization of nuclear encoded r-proteins in plants. However, mitochondrial r-protein localization has been studied in *Drosophila* (Frei et al., 2005), mice (Chen et al., 2007) and yeast (Saveanu et al., 2001) using tagged-proteins and appears to be an effective tool to study subcellular localization of the plant type II RPS15a r-proteins.

It is likely that RPS15aB and -E have evolved from type I RPS15as, acquiring mitochondrial targeting signals, to be incorporated into mitochondrial ribosomes. Classically, mitochondrial targeted proteins interact with mitochondrial import receptors via a cleavable N-terminus comprised of positively charged and hydrophobic amphipathic helices (Hansel et al., 2002). While mitochondrial localization signals share very little amino acid identity, the N-termini of type II RPS15as and RPS8 (its N-terminus is an RNA binding domain and not a localization signal) contain some degree of conservation, however it seems likely that the mitochondrial localization signal is intrinsic to the protein amino acid sequence, as the N-terminus is more similar between the two clades of RPS15a than to RPS8. This does not rule out the possibility that RPS15aB and RPS15aE utilize their N-termini as a localization signal and a RNA binding domain. Site-directed mutagenesis (Ling and Robinson, 1997) of the N-terminus of RPS15aB and RPS15aE would shed some light on this possibility. It has been estimated that approximately 75% of nuclear-encoded plant mitochondrial r-proteins have N-terminal extensions or, the N-terminus is longer than their prokaryotic ortholog (Bonen and Calixte, 2006). However, RPS15aB and RPS15aE lack an N-terminal extension (Adams et al., 2002) suggesting that the mitochondrial targeting sequence could lie elsewhere within the protein. If all RPS15a isoforms utilize the N-

terminus for their differential cellular localization, it is likely that the 24/40 residue differences (highlighted in Figure 3.1) account for their nucleolus versus mitochondrial localization patterns. In addition, RPS15aB and RPS15aE lack a confirmed yeast S22 monopartite nuclear localization signal, GKRQVLIRP (Timmers et al., 1999) found in all Arabidopsis type I cytosolic RPS15as and other related type I RPS15as (Figure 3.1) which likely accounts for their differences in localization. The lack of this nuclear localization signal suggests that RPS15aE would not interact with cytoplasmic 18S rRNA *in vivo*, and that its highly conserved putative RNA binding domain (Figure 3.1) is responsible for the binding to cytoplasmic 18S rRNA *in vitro*.

Previous results had suggested that type II divergent copies of RPS15a were not only imported into flowering plant mitochondria (Adams et al., 2002), but were also incorporated into mitochondrial ribosomes (Carroll et al., 2007). However, recent mass spectrometry analysis of Arabidopsis cytoplasmic ribosomes has resulted in some confusion concerning the location of the type II RPS15as. Both type I RPS15aA/F and type II RPS15aE were shown to be incorporated in cytoplasmic ribosomes (Chang et al., 2005), however, recent analysis has determined that RPS15aE is not found in the cytoplasmic ribosome (Carroll et al., 2007). Results from my work (section 3.2 & 3.6) support those of Carroll et al. (2007) in that while RPS15aE is able to bind to cytoplasmic 18S rRNA, it is the only r-protein (of the subset studied) that was able to interact with mitochondrial 18S rRNA (Figure 3.6 B & 3.11), suggesting that the type II RPS15a r-proteins, RPS15aB and RPS15aE, have functionally replaced RPS8 in the plant mitochondria, and should therefore be considered RPS8 homologs. Once the incorporation of RPS15aB and RPS15aE into plant mitochondrial ribosomes is confirmed, the debate concerning the location and function of type II RPS15aB and RPS15aE will likely be closed.

Molecular phylogenetics suggests the RPS15a divergence occurred fairly late in land plant evolution as the liverwort, *Marchantia polymorpha*, contains the *RPS8* gene within its mitochondrial genome (Adams et al., 2002). In Arabidopsis, as well as RPS15a, there are two other r-protein families, RPP2 and RPL7, that also show evolutionary distinct inter-family variation (Chang et al., 2005). Both RPS15a and RPL7 divergence is thought to have occurred prior to the divergence of monocots and eudicots, whereas the RPP2 gene family variation appears to have occurred after this divergence (Chang et al., 2005).

4.3. RPS15a isoforms are probable primary rRNA binders

In prokaryotes, all primary binding proteins bind 16S rRNA, however not all proteins that bind 16S rRNA are primary binding proteins; some proteins that bind naked rRNA are secondary binders. Upon rRNA binding, primary binders induce conformational changes to allow secondary r-protein binding. For this reason it is impossible through EMSA analysis to definitively say that RPS15a is a primary binding protein, although the evidence provided in this thesis suggests it is. In addition, some of the EMSA shifts could be attributed to conformational changes occurring to the rRNA molecules upon RPS15a binding. For RPS15aA/F, all of the RPS15a isoforms used in these binding studies bound full length cytoplasmic 18S rRNA or mitochondrial 18S rRNA (RPS15aE only) independently, without the prior association of any other r-proteins with the rRNA, supporting the notion of RPS15a as a primary binding r-protein. As the largest shifts corresponded to the lanes containing an extreme excess of RPS15a, some of the interactions may be non-specific. However, if RPS15a functions as a primary binder of helix 23 of 18S rRNA would likely reflect the high degree of conservation of r-protein binding-rRNA interactions for the S8p family.

R-protein-rRNA binding is dependent on the presence of at least one rRNA binding domain within the protein and its complementary rRNA binding site. RPS8 contains two RNA binding domains, one within the C-terminus and one within the N-terminus of the polypeptide (Wower et al., 1992) whereas RPS15a appears to only contain one RNA binding domain. As described previously, the putative C-terminal RNA binding domain of RPS15a is highly conserved in both type I and type II isoforms: -D and -E, 63%, -A and -E, 70% identity. While this level of identity supports the suggestion that this region is an RNA binding domain for RPS15a, site-directed mutagenesis would be required to confirm this. It is tempting to speculate that if the C-terminus of RPS15a is occupied by 18S rRNA, then perhaps regions situated within the N-terminus, which are more negatively charged, could be interacting with other proteins. Support for this speculation comes from the prokaryotic ortholog RPS8 which interacts with two other r-proteins (RPS5 and RPS21) during 30S small subunit assembly (Jagannathan and Culver, 2003). In *E. coli*, amino acid residues 90-96 of RPS8 (Nevskaya et al., 1998) interact most extensively with the C-terminus (residues 70-90 and 140-155) of RPS5 (Brodersen et al., 2002). While the level of sequence identity of the putative RNA binding domains of all RPS15a isoforms is relatively high, differences with

respect to prokaryotic RPS8 are present. Type I RPS15as share approximately 32% amino acid identity and 64% amino acid similarity with the confirmed C-terminal rRNA binding domain of RPS8 whereas type II RPS15as share 20% amino acid identity and 55% amino acid similarity with the C-terminal RNA binding domain of RPS8. Although type I RPS15as share an overall higher amino acid identity with prokaryotic RPS8 compared to type II RPS15as, the rRNA binding domains of type II proteins have likely evolved from that of type I proteins (Chang et al., 2005) to accommodate binding to mitochondrial 18S rRNA, a closer relative of prokaryotic 16S rRNA. As the RNA binding domain of RPS8 shares less amino acid identity with RPS15aB and RPS15aE than type I RPS15as, it is probable that the structure of type II RPS15a's RNA binding domain plays a larger role in binding to 18S mitochondrial rRNA than its amino acid sequence.

R-protein-RNA binding can be best analyzed via X-ray crystallography. Experimental data suggests that r-protein interactions with RNA are likely to occur through basic residues (such as those found at the C-terminus of RPS15a) contacting the negatively charged sugar phosphate backbone of the RNA, or through aromatic residues, participating in stacking interactions with RNA bases (Liljas and Al-Karadaghi, 1997). A common structural feature of RNA binding domains is the presence of β -sheet surfaces at the primary site of RNA recognition. The RNA binding domain of RPS8 is composed of 6 β -sheets (Davies et al., 1996; Brodersen et al., 2002). RPS15a protein secondary structural predictions (modeled using Rasmol v2.6 Beta-2a application) are in agreement with the confirmed C-terminal structure of RPS8, as β -sheets make up the core of its putative RNA binding domain. While these data were produced from *in vitro* studies, the experimental conditions used for binding mimicked the plant cell physiological pH (ranging pH 7.2-8 depending on tissue type) and ion concentrations, suggesting that the RPS15a-rRNA interactions occur *in vivo*.

Although type I and type II RPS15as can bind to cytoplasmic 18S rRNA, differences in primary protein structure between the two clades probably reflect their different binding preferences for cytoplasmic versus mitochondrial rRNA as well as other r-proteins, eukaryotic versus mitochondrial.

4.4. Differential binding preferences of evolutionary divergent RPS15a isoforms

There is now evidence to suggest that the ribosome is not only responsible for protein synthesis, it is also responsible for mRNA transcript selection, likely facilitated through ribosome heterogeneity (Vanderhaeghen et al., 2006). Ribosome heterogeneity (of r-proteins) has been observed in many plant ribosomes (Chang et al., 2005; Giavalisco et al., 2005; Carroll et al., 2007), roundworm ribosomes (RPS19; Etter et al., 1996), yeast ribosomes (P-stalk; Garcia-Marcos et al., 2008), and slime mold ribosomes (several r-proteins; Ramagopal, 1992), leading to scientific speculation on the functional roles of such diversity.

Unlike the P-protein stalk, composed of four acidic P-proteins, P0, P1, P2 and P3 (Carroll et al., 2007), where differential expression patterns have been observed for the different isoforms of each protein (Szick-Miranda and Bailey-Serres, 2000), expression patterns and transcript abundance for Arabidopsis type I RPS15as are relatively similar (Hulm et al., 2005). The high amino acid sequence identity shared among RPS15as, particularly at the putative C-terminal rRNA binding domain, led to my initial hypothesis that all of the RPS15as tested would bind cytoplasmic 18S rRNA. However, the differences in RPS15a binding affinity to different fragments of the 18S rRNA helix 23 (section 3.8 & 3.9) prompt the question, why do different type I RPS15a isoforms, (98-100% amino acid identity) have different binding characteristics for different regions of 18S rRNA helix 23? Prior to answering this question, a second question, how do type I RPS15as differentiate between different regions of the 18S rRNA helix 23, needed to be addressed.

Few studies have been carried out to identify whether eukaryotic r-proteins bind more than one RNA site (i.e. two different RNA helices or mRNA and rRNA) although in yeast, RPS14 was shown to bind mRNA and rRNA (Fewell and Woolford, 1999). My research suggests that RPS15aD and RPS15aE, unlike RPS15aA/F, can bind multiple rRNA targets, and it is possible that RPS15aE may contain more than one rRNA binding domain. In prokaryotes, r-proteins generally contain more than one RNA binding domain, which allows these r-proteins to bind independently to two or more distinct sites on rRNA. Another example is human RNA binding protein U1A that binds hairpin structures stem loop I (Query et al., 1989), stem loop II (Scherly et al., 1989) and an internal loop (van Gelder et al., 1993) of U1 snoRNA. It is possible that some RPS15a isoforms bind more than one RNA site.

While more than one RNA binding domain in RPS15aE appears plausible, more than one RNA binding domain in RPS15aD seems unlikely due to its high amino acid sequence similarity with RPS15aA/F (Figure 3.1- 2 amino acids different). The first substitution, an A¹⁰→G is a conserved substitution, whereas the second substitution, Y¹⁰¹→F, for RPS15aD, results in the loss of the tyrosine OH group resulting in the loss of a potential phosphorylation site. Although these experiments were conducted *in vitro*, the *E. coli* genome contains genes for tyrosine kinases that can phosphorylate tyrosine residues of eukaryotic proteins (Kenny and Finlay, 1997; Rosenshine et al., 1996), as well as phosphatases that structurally resemble those found in eukaryotes (Kennelly, 2002). In this case, prokaryotic expressed RPS15aA/F may be phosphorylated, altering the protein's conformation and activity. This could be tested experimentally by Western blot analysis of the purified RPS15aA/F r-protein (that was expressed in *E. coli*) using an antibody against phosphorylated tyrosine (P-Tyr), which is specific enough to detect one phosphorylated tyrosine residue (Sickmann and Meyer, 2001). It has been suggested that regions closest to a phosphorylated amino acid undergo the most significant structural changes (Groban et al., 2006): in this case Y/F¹⁰¹ is within the putative rRNA binding domain of RPS15aA/F and RPS15aD (Figure 3.1). Phosphorylation can modulate how a protein interacts with nucleic acids, or other proteins. It is possible that the RNA binding domains of RPS15aA/F and RPS15aD may differ because of this Y¹⁰¹→F substitution, which could modulate affinity for 18S rRNA, and provide some explanation for the differential binding characteristics observed. Lisitsky and Schuster (1995) reported that the phosphorylation of a single serine residue within the RNA binding domain of a RNA-binding protein, in chloroplasts of spinach, changed its affinity for RNA. However, Carroll et al. (2007) did not identify any RPS15a isoforms as candidates for phosphorylation, although additional phosphorylation analyses would confirm or refute this possibility.

For RPS15aE, there remains the possibility of a second RNA binding domain, allowing RPS15aE to recognize all of the cytoplasmic helix 23 loop variants, as well as mitochondrial 18S rRNA. RNA binding domains interacting with multiple rRNA structures can be associated with a diversity of biological functions (Varani and Nagai, 1998). The presence, at three different positions within the C-terminus, of non-conserved amino acids could

account for the ability of RPS15aE to bind both cytoplasmic and mitochondrial 18S rRNA. At the C-terminus, the type II RPS15as contain the substitutions A¹⁰⁸→D, R¹¹⁷→I, K¹²⁴→Q, all involving changes in charge (minus two positive, plus one negative) and could affect binding affinity for 18S rRNA. In addition, other amino acids outside of the putative C-terminal RNA binding domain may impact tertiary structure and subsequent RNA recognition. It is important to note that *in vivo*, mitochondrial targeted RPS15aE would likely not encounter cytoplasmic 18S rRNA.

With respect to helix 23 binding by type I RPS15a, I am still left asking the question, why would RPS15a isoforms differentially bind different regions of the 18S rRNA helix 23? Although there is the possibility that RPS15aA/F or -D preferentially bind different regions of 18S rRNA, or that they require other helices in addition to helix 23 for optimal binding, it is also possible that type I RPS15a isoforms bind different 18S rRNA helix 23 junctions, inducing slight conformational changes to 18S rRNA, resulting in endless possible effects on downstream processes. Although purely speculative, there could be some interesting consequences to the platform domain of 18S rRNA upon r-protein isoform binding. For example, changes in central (platform) rRNA domain structure could affect translation efficiency, as reported in 16S rRNA (Vila et al., 1994). Alterations to two nucleotides (571, 865; *E. coli* numbering) in the platform domain resulted in dramatically impaired translation efficiency of all mRNAs and structural stability of 16S rRNA (Vila et al., 1994). Changes in rRNA structure resulting from different isoform binding could subsequently affect secondary and/or tertiary r-protein binding, as demonstrated with RPS15, a primary binder, which binds a three way helix junction in 16S rRNA (Orr et al., 1998), inducing a conformational change that allows the subsequent binding of RPS6 and RPS18 (Agalarov et al., 2000). If RPS15 is not present to bind these helical junctions, differences in inter-helical angles affecting the conformation of 16S rRNA were observed, directly affecting the binding of other r-proteins (Agalarov et al., 2000).

Overall, cytoplasmic and mitochondrial 18S rRNA sequences share 42.4 % nucleotide identity, with a similar level of nucleotide identity (41.8%) between the protein binding sites. At the amino acid level, type I and type II RPS15as are most similar at the C-terminus. S8p members all contain similar putative RNA binding domains, a structural feature that has been conserved over the course of time. RPS8 and type II RPS15as share

similarities at the C-terminal amino acid sequence, the predicted secondary structures of type II RPS15as are very similar to that of the resolved RNA binding domain of RPS8, suggesting that the RNA binding domain's structure likely plays a key role in rRNA interactions/binding.

4.5. The rRNA binding site(s) of RPS15a

To date, the 16S rRNA binding site of RPS8 is one of the best characterized of all r-proteins. This interaction between RPS8 and 16S rRNA helix 21 is necessary for 30S subunit assembly. This and other prokaryotic r-protein-rRNA interactions are thought to be conserved in eukaryotes. Based on the nucleotide sequence of helix 21 (to which RPS8 binds) a larger, more structurally diverse helix 23 (Figure 3.16) was identified as the best candidate for RPS15a binding. Due to the lack of crystal structures, the 18S rRNA secondary structure (Figure 1.1) from which the helix 23 structure (Figure 3.16) is derived, could in reality be different to the predicted structure.

Using EMSAs, I investigated the probable 18S rRNA binding site, helix 23, for RPS15a binding. All of the RPS15a isoforms interacted with at least one loop fragment of helix 23, however, from my binding studies, helix 23 loop 4-6 emerged as a prime candidate for the RPS15a binding site. It also contains a high degree of sequence identity with the 16S rRNA binding site for RPS8 (Figure 3.16). Specifically, RPS15aD and RPS15aE were able to bind the entire helix 23 18S rRNA loop fragment, and its sub-loop fragments, whereas RPS15aA/F bound only loop 4-6 (Figure 3.8). Although it seems inconsistent that RPS15aA/F does not bind the entire helix 23, it is possible that the entire helix 23 was folded slightly differently than loop 4-6, and that this alteration may affect RPS15aA/F binding. The specificity of the interactions was confirmed with competition EMSAs using RPS15a mRNAs as semi-specific competitors. As each mRNA contains similar elements to the helix 23 18S rRNA, a high concentration of mRNA was able to compete for RPS15a binding, except for RPS15a binding to loop 4-6. This implies that interactions between the different RPS15a isoforms and 18S rRNA helix 23 loop fragment 4-6 are specific. Supershift assays further confirmed loop 4-6 specificity. Although some differential binding was observed between the RPS15a isoforms, these results suggest that RNA binding sites and likely RNA-protein interactions for RPS8 and RPS15a have been conserved over the course of evolution.

Although the binding site for RPS15aE on mitochondrial 18S rRNA was not investigated, the structural similarity between helix 21 of *E. coli* 16S rRNA and helix 21 of Arabidopsis mitochondrial 18S rRNA cannot be ignored, and until further investigation, appears to be the best candidate as the RPS15aE rRNA binding site. There is a growing body of evidence to suggest that r-proteins recognize rRNA primarily based on unique secondary structure rather than primary sequence, as discussed previously for RPL11 (section 1.4). Results from mRNA transcript EMSAs appear to agree with this suggestion. Primary sequence alignments between RPS15a mRNAs, helix 21 of 16S rRNA, and helix 23 of cytoplasmic 18S rRNA identified regions of conservation, suggesting that RPS15a may be able to bind to its own mRNA, as well as rRNA, in a manner similar to some prokaryotic r-proteins, where mRNA-binding sites often show structural and/or sequence similarities to rRNA binding sites (Nomura et al., 1980; Draper, 1989; Zengel and Lindahl, 1994). Although mRNA and 18S rRNA helix 23 sequences displayed a moderate level of similarity, several RNA secondary structural prediction programs (entire mRNA: RNA fold; <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>, RNA secondary structure prediction; http://www.genebee.msu.su/services/rna2_reduced.html, Sfold; <http://sfold.wadsworth.org>) predicted loops structures within the mRNA molecules that did not resemble any of the 18S rRNA helices. It is possible therefore that although the RPS15a mRNA could contain elements of the RPS15a binding sites, they are structurally unique and are not recognized by any of the RPS15a isoforms. A lack of RPS15a binding suggests that RPS15a expression is not governed by competitive binding between 18S rRNA and RPS15a mRNA.

No RPS15a isoforms interacted with either ssDNA or dsDNA corresponding to helix 23 of 18S rRNA. Despite the fact RPS8 does not bind DNA, at its N-terminus, RPS8 contains an $[\alpha]-[\beta]-[\alpha]-[\beta]-[\beta]$ fold, also found in the DNA binding proteins DNase I and *HaeIII* methyltransferase (Davies et al., 1996). A lack of structural information for RPS15a allowed us to speculate that this motif may be conserved in RPS15a isoforms, however, the rDNA sequences were not recognized by either type I RPS15aA/F or type II RPS15aE. Thus if a similar structural motif is still present in RPS15a, it has been modified to bind RNA instead of DNA.

The sequence comparisons conducted in this research suggest that the RNA binding domains of both type I and type II RPS15as are very similar and that the corresponding

binding sites (helix 21 [16S rRNA or mitochondrial 18S rRNA] or helix 23 [cytoplasmic 18S rRNA]) on small subunit rRNA are also conserved. However, RPS8 binds at two sites on 16S rRNA utilizing at least 3 major regions of the entire protein, amino acids 4-17, 76-80, and 105-123. Therefore the possibility exists that RPS15a binds to multiple sites on 18S rRNA. Although this research cannot rule out the possibility of multiple 18S rRNA binding sites, alignment data demonstrates that the highest degree of conservation between RPS15a and RPS8 lies at the C-terminus, with the other two RNA binding domains of RPS8 not appearing to be conserved. Future experiments probing the structure of the Arabidopsis 40S ribosomal subunit will confirm the location of RPS15a on 18S rRNA whereas studying the 40S ribosomal subunit assembly will identify the number and location of rRNA binding sites. However, a more simplistic preliminary experiment involving the mutation or alteration of helix 23 of full length 18S rRNA followed by subsequent binding reactions with RPS15a would also shed light on whether or not helix 23 of 18S rRNA is the only binding site for RPS15a.

Two distinct base modifications occur within helix 23 of 18S rRNA. Although no specific functional role has been attributed to each modification, specific structural changes to rRNA may result (such as altered steric properties), therefore, it is likely that functional roles also exist, but are as yet poorly understood. For example, a possible role for pseudouridines include rRNA structural stabilization (Arnez and Steitz, 1994) via rigidifying the sugar-phosphate backbone and enhancing base stacking (Charette and Gray 2000). Because the distribution of modified nucleotides does not appear to be random, and because helix 23 of the central domain is not in proximity to heavily modified sites within the small subunit (A-site, P-site, E-site, mRNA latch, helix 44, inter-subunit bridge), it can be suggested that base modification within helix 23 does not play a major role in RPS15a binding. However, U763 is in close proximity to the “conserved core” nucleotides of helix 23’, and could potentially play a role in RPS15a binding. Modification of this nucleotide followed by EMSAs with RPS15a isoforms would shed light on this possibility.

4.6. Conclusions and the future of ribosomal protein S15a research

In this study, I examined the binding of expressed Arabidopsis RPS15aA/F, -D and

-E to cytoplasmic 18S rRNA, mitochondrial 18S rRNA, cytoplasmic 18S rRNA loop fragments, RPS15a mRNA and both ds/ss rDNA. Like RPS8 binding to 16S rRNA, I have demonstrated that all RPS15a isoforms are able to bind directly to cytoplasmic 18S rRNA. This is the first time RPS15a has been mapped to 18S rRNA. These results support the hypothesis that RPS15a is a functional homologue of *E.coli* r-protein RPS8. Furthermore, the *in vivo* localization of RPS15aE to mitochondria, its binding to mitochondria 18S rRNA, and the sequence conservation between RPS8/RPS15a r-proteins and their putative rRNA binding sites strongly suggests that RPS15aE has replaced RPS8 in Arabidopsis mitochondria. My results are consistent with a primary 18S rRNA binder role for RPS15a, however, RPS15a incorporation into functional polysomes still needs to be confirmed.

It is interesting that cytosolic RPS15aA/F and RPS15aD interact with cytoplasmic 18S rRNA as the significance of more than one r-protein isoform in plants has yet to be determined. It is likely that both cytosolic RPS15a isoforms are incorporated into ribosomal small subunits, as they are targeted to the nucleolus (J. Hulm, Ph.D thesis) and bind 18S rRNA. With respect to the ribosome filter hypothesis (Mauro and Edelman, 2002) it is possible that different RPS15a isoform ribosomes could display differential affinity for binding specific export factors or mRNAs.

In the end, my project has left me with still many questions to be answered regarding plant r-proteins. Our understanding of plant r-protein transcriptional, post transcriptional, translational and post translation regulation, the coordinated expression of multiple r-protein genes and the requirement for multiple expressed r-protein isoforms is minimal. This project investigated the binding preferences of RPS15a isoforms and determined a putative rRNA binding site, laying the foundation for future work to determine precisely how RPS15a binds small subunit rRNA.

Future experiments should confirm RPS15a incorporation into functional polysomes. Experimentally, incorporation of RPS15a into functional polysomes could be attempted by generating transgenic Arabidopsis expressing a C-terminally FLAG-His6 tagged RPS15a and immunopurifying intact polysomes with anti-FLAG agarose beads (Zanetti et al., 2005). Furthermore, immunoaffinity purification of polyribosomes could also be used to compare the mRNAs associated with polysomes containing either RPS15aA/F or RPS15aD. This

comparison could identify any possible functional significance of RPS15a heterogeneity and selective translation.

With respect to RPS15a-rRNA binding, ideally future experiments would study the structure of the r-protein isoform-rRNA complexes. X-ray crystal analysis of RPS15a isoforms bound to 18S rRNA would highlight any unique isoform-rRNA interactions by identifying the actual rRNA domain for RPS15a binding and the exact rRNA helices involved in this interaction. As well as X-ray crystallography analysis, NMR could also be used to highlight this interaction. Footprinting and probing (chemical and/or enzymatic reactions, Brenowitz et al., 1986, or hydroxyl radical footprinting, Dixon et al., 1991) studies could also be useful in probing this r-protein-rRNA relationship.

In eukaryotes, it is assumed that early assembly r-proteins bind to pre-rRNA (Gerbi and Borovjagin, 2004) in addition to mature rRNA. In yeast, often assembly r-proteins are found to co-precipitate with the SSU processome (which is required for 18S rRNA biogenesis; Saez-Vasquez et al., 2004), and it would be interesting to investigate whether RPS15a is apart of the early stages of small subunit biogenesis in plants. Identifying an association of RPS15a with 18S pre-rRNA or any interactions with the SSU processome complex would also support the suggestion that RPS15a is an early binding r-protein.

REFERENCES

- Adams, K.L., Daley, D.O., Whelan, J., Palmer, J.D. (2002) Genes for two mitochondrial ribosomal proteins in flowering plants are derived from their chloroplast or cytosolic counterparts. *Plant Cell* 14: 931-943.
- Agalarov, S.C., Zheleznyakova, E.N., Selivanova, O.M., Zheleznyaya, L.A., Matvienko, N.I., Vasiliev, V.D., Spirin, A.S. (1998) *In vitro* assembly of a ribonucleoprotein particle corresponding to the platform domain of the 30S ribosomal subunit. *Proc. Natl. Acad. Sci. USA* 95: 999-1003.
- Agmon, I., Auerbach, T., Baram, D., Bartels, H., Bashan, A., Berisio, R., Fucini, P., Hansen, H.A., Harms, J., Kessler, M., Peretz, M., Schlutzen, F., Yonath, A., Zarivach, R. (2003) On peptide bond formation, translocation, nascent protein progression and the regulatory properties of ribosome. *Eur. J. Biochem.* 270: 2543-56.
- Ali, A.B., Wuyts, J., De Wachter, R., Meyer, A., Van de Peer, Y. (1999) Construction of a variability map for eukaryotic large subunit ribosomal RNA. *Nucl. Acids Res.* 27: 2825-2831.
- Aliprandi, P., Sizun, C., Perez, J., Mareuil, F., Caputo, S., Leroy, J.L., Odaert, B., Laalami, S., Uzan, M., Bontems, F. (2008) S1 ribosomal protein functions in translation initiation and ribonuclease RegB activation are mediated by similar RNA-protein interactions: an NMR and SAXS analysis. *J. Biol. Chem.* 283: 13289-13301.
- Alkemar, G. and Nygård, O. (2006) Probing the secondary structure of expansion segment ES6 in 18S ribosomal RNA. *Biochemistry* 45: 8067-8078.
- Allas, Ü., Liiv, A., Remme, J. (2003) Functional interaction between RNase III and the *Escherichia coli* ribosome. *BMC Mol. Biol.* 4: 8.
- Arkov, A.L., A. Mankin, E.J. Murgola (1998) An rRNA fragment and its antisense can alter decoding of genetic information. *J. Bacteriol.* 180: 2744-2748.
- Arndt, K.M. and Kane, C.M. (2003) Running with RNA polymerase: eukaryotic transcript elongation. *Trends Genet.* 19: 543-550.
- Arnez, J.G. and Steitz, T.A. (1994) Crystal structure of unmodified tRNA (Gln) complexed with glutaminyl-tRNA synthetase and ATP suggests a possible role for pseudo-uridines in stabilization of RNA structure. *Biochemistry* 33: 7560-7567.

- Arnold, R. J. and Reilly, J. P. (1999) Observation of *Escherichia coli* ribosomal proteins and their posttranslational modifications by mass spectrometry. *Anal. Biochem.* 269: 105-112.
- Ban, N., Nissen, P., Hansen, J., Moore, P.B., Steitz, T.A. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* 289: 905-920.
- Barakat, A., Szick-Miranda, K., Chang, I-F., Guyot, R., Blanc, G., Cooke, R., Delseny, M., Bailey-Serres, J. (2001) The organization of cytoplasmic ribosomal protein genes in the *Arabidopsis* genome. *Plant Phys.* 127: 398-415.
- Barneche, F., Steinmetz, F., Echeverria, M. (2000) Fibrillarin genes encode both a conserved nucleolar protein and a novel small nucleolar RNA involved in ribosomal RNA methylation in *Arabidopsis thaliana*. *J. Biol. Chem.* 275: 27212-27220.
- Barta, A., Steiner, G., Brosius, J., Noller, H.F., Kuechler, E. (1984) Identification of a site on 23S ribosomal-RNA located at the peptidyl transferase center. *Proc. Natl. Acad. Sci. USA* 81: 3607-3611.
- Barth-Baus, D., Stratton, C.A., Parrott, L., Myerson, H., Meyuhas, O., Templeton, D.J., Landreth, G.E., Hensold, J.O. (2002) S6 phosphorylation-independent pathways regulate translation of 5'-terminal oligopyrimidine tract-containing mRNAs in differentiating hematopoietic cells. *Nucl. Acids Res.* 30: 1919-1928.
- Bautista- Zapanta, J., Suzuki, K., Yoshida, K. (2002) Characterization of four ribosomal RNA operons in the genome of *Agrobacterium tumefaciens*. *Nucl. Acids Res.* 2: 91-92.
- Bernstein, K., Gallagher, J.E., Mitchell, B.M., Granneman, S., Baserga, S.J. (2004) The small-subunit processome is a ribosome assembly intermediate. *Eukaryot. Cell* 3: 1619-1626.
- Binder, S., Brennicke, A. (2003) Gene expression in plant mitochondria: transcriptional and posttranscriptional control. *Phil. Trans. R. Soc. Lond. B.* 358: 181-189.
- Bohman, K., Ruusala, T., Jelenc, P.C., Kurland, C.G. (1984) Kinetic impairment of restrictive streptomycin-resistant ribosomes. *Mol. Gen. Genet.* 198: 90-99.

- Bonham-Smith, P.C. and Moloney, M.M. (1994) Nucleotide and protein sequences of a cytoplasmic ribosomal protein S15a gene from *Arabidopsis thaliana*. *Plant Physiol.* 106: 401-402.
- Bonham-Smith, P.C. and Bourque, D.P. (1990) The chloroplast genome and regulation of its expression. In: K.W. Adolph (Ed.), *Chromosomes: Eukaryotic, Prokaryotic and Viral*. CRC Press, Boca Raton, FL, pp. 179-261.
- Blanc, G., Barakat, A., Guyot, R., Cooke, R., Delseny, M. (2000) Extensive duplication and reshuffling in the *Arabidopsis* genome. *Plant Cell* 12: 1093-1102.
- Blyn, L.B., Risen, L.M. Griffey, R.H., Draper, D.E. (2000) The RNA-binding domain of ribosomal protein L11 recognizes an rRNA tertiary structure stabilized by both thiostrepton and magnesium ion. *Nucl. Acids Res.* 28: 1778-1784.
- Bonen, L. and Calixte, S. (2006) Comparative analysis of bacterial-origin genes for plant mitochondrial ribosomal proteins. *Mol. Biol. Evol.* 23: 701-712.
- Brandizzi, F., Hanton, S., DaSilva, L.L., Boevink, P., Evans, D., Oparka, K., Denecke, J., Hawes, C. (2003) ER quality control can lead to retrograde transport from the ER lumen to the cytosol and the nucleoplasm in plants. *Plant J.* 34: 269-281.
- Brenowitz, M., Senear, D. F., Shea, M. A., Ackers, G. K. (1986) Quantitative DNase footprint titration: a method for studying protein-DNA interactions. *Methods Enzymol.* 130: 132-181.
- Brett, D., Pospisil, H., Valcárcel, J., Reich, J., Bork, P. (2002) Alternative splicing and genome complexity. *Nat. Genet.* 30: 29-30.
- Brodersen, D.E., Clemons Jr., W.M., Carter, A.P., Wimberly, B.T., Ramakrishnan, V. (2002) Crystal structure of the 30S ribosomal subunit from *Thermus thermophilus*: Structure of the proteins and their interactions with 16 S RNA. *J. Mol. Biol.* 316: 725-768.
- Brodersen, D.E. and Nissen, P. (2005) The social life of ribosomal proteins. *FEBS J.* 272: 2098-2108.
- Brown, J.W.S. and Shaw, P.J. (1998) Small nucleolar RNAs and pre-rRNA processing in plants. *Plant Cell.* 10: 649-658.
- Bycroft, M., Hubbard, T.J.P., Proctor, M., Freund, S.M.V., Murzin, A.G. (1997) The solution structure of the S1 RNA binding domain: a member of an ancient nucleic acid-binding fold. *Cell* 88: 235-242.

- Caburet, S., Conti, C., Schurra¹, C., Lebofsky, R., Edelstein, S.J., Bensimon, A. (2005) Human ribosomal RNA gene arrays display a broad range of palindromic structures. *Gen. Res.* 15: 1079-1085
- Carroll, A.J., Heazlewood, J.L., Ito, J., Millar, A.H. (2007) Analysis of the Arabidopsis cytosolic ribosome proteome provides detailed insights into its components and their post-translational modification. *Mol. Cell Proteomics* 7: 347-69.
- Cech, T. R. (2000) The ribosome is a ribozyme. *Science* 289: 878-879.
- Cerretti, D.P., Mattheakis, L.C., Kearney, K.R., Vu, L., Nomura, M. (1988) Translational regulation of the *spc* operon in *Escherichia coli*: Identification and structural analysis of the target site for S8 repressor protein. *J. Mol. Biol.* 204: 309-329.
- Chandramouli, P., Topf, M., Ménétret, J-F., Eswar, N., Cannone, J.J., Gutell, R.R., Sali, A., Akey, C.W. (2008) Structure of the mammalian 80S ribosome at 8.7 Å resolution. *Structure* 16: 535-548.
- Chang, I-F., Szick-Miranda, K., Pan, S., and Bailey-Serres, J. (2005) Proteomic characterization of evolutionarily conserved and variable proteins of Arabidopsis cytosolic ribosomes. *Plant Phys.* 137: 848-862.
- Chao, S., Sederoff, R., Levings, C.S. (1984) Nucleotide sequence and evolution of the 18S ribosomal RNA gene in maize mitochondria. *Nucl. Acids Res.* 12: 6629-6644.
- Charette, M. and Gray, M.W. (2000) Pseudouridine in RNA: what, where, how, and why. *IUBMB Life* 49: 341-351.
- Chavez-Rios, R., Arias-Romero, L.E., Almaraz-Barrera, J., Hernandez-Rivas, R., Guillen, N., Vargas, M. (2003) L10 ribosomal protein from *Entamoeba histolytica* share structural and functional homologies with QM/Jif-1: proteins with extraribosomal functions. *Mol. Biochem. Parasitol.* 127: 151-60.
- Chen, D. and Huang, S. (2001) Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. *J. Cell Biol.* 153: 169-176.
- Chen, Y., Chang, M., Shiau, A., Yo, Y., Wu, C. (2007) Mitochondrial ribosomal protein S36 delays cell cycle progression in association with p53 modification and p21 expression. *J. Cell. Biochem.* 100: 981-990.

- Chiaruttini, C., Milet, M., de Smit, M., Springer, M. (1996) Translational coupling in the *Escherichia coli* operon encoding translation initiation factor IF3 and ribosomal proteins L20 and L35. *Biochimie* 78: 555-567.
- Chowdhury, S., Pal, S., Ghosh, J., DasGupta, C. (2002) Mutations in domain V of the 23S ribosomal RNA of *Bacillus subtilis* that inactivate its protein folding property *in vitro*. *Nucl. Acids Res.* 30: 1278-1285.
- Correll, C.C., Munishkin, A., Chan, Y.L., Ren, Z., Wool, I.G., Steitz, T.A. (1998) Crystal structure of the ribosomal RNA domain essential for binding elongation factors. *Proc. Natl. Acad. Sci. USA* 95: 13436-13441.
- Cuccurese, M., Russo, G., Russo, A., Pietropaolo, C. (2005) Alternative splicing and nonsense-mediated mRNA decay regulate mammalian ribosomal gene expression. *Nucl. Acids Res.* 33: 5965–5977.
- Dai Z., Gao J., An K., Lee J., Edwards, G.E. (1996) Promoter elements controlling developmental and environmental regulation of a tobacco ribosomal protein gene *L34*. *Plant Mol. Biol.* 32: 1055-1065.
- Davies, C., Ramakrishnan, V., White, S.W. (1996) Structural evidence for specific S8–RNA and S8–protein interactions within the 30S ribosomal subunit: ribosomal protein S8 from *Bacillus stearothermophilus* at 1.9 Å resolution. *Structure* 4: 1093-1104.
- Dechampesme, A-M., Koroleva, O., Leger-Silvestre, I., Gas, N., Camier, S. (1999) Assembly of 5S ribosomal RNA is required at a specific step of the pre-rRNA processing pathway. *J. Cell Biol.* 145: 1369-1380.
- Degenhardt, R.F. and Bonham-Smith, P.C. (2008) Arabidopsis ribosomal proteins RPL23aA and RPL23aB are differentially targeted to the nucleolus and are disparately required for normal development. *Plant Physiol.* 147: 128-142.
- Della Seta, F., Ciafré, S.A., Marck, C., Santoro, B., Presutti, C., Sentenac, A., Bozzoni, I. (1990) The ABF1 factor is the transcriptional activator of the *L2* ribosomal protein genes in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 10: 2437-2441.
- Dinman, J.D. (2005) 5S rRNA: Structure and function from head to toe. *Int. J. Biomed. Sci.* 1: 2-7.
- Dixon, W.J., Hayes, J.J., Levin, J.R., Weidner, M.F., Dombroski, B.A., Tullius, T.D. (1991) Hydroxyl radical footprinting. *Methods Enzymol.* 208: 380-413.

- Doelling, J.H., Gaudino, R.J., Pikaard, C.S. (1993) Functional analysis of *Arabidopsis thaliana* rRNA gene and spacer promoters *in vivo* and by transient expression. Proc. Natl. Acad. Sci. USA 90: 7528-7532.
- Doelling, J.H. and Pikaard, C.S. (1995) The minimal ribosomal RNA gene promoter of *Arabidopsis thaliana* includes a critical element at the transcription initiation site. Plant J. 8: 683-692.
- Donovan, D.M. and Pearson, N.J. (1986) Transcriptional regulation of ribosomal proteins during a nutritional upshift in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6: 2429-2435.
- Draper, D.E. (1989) How do proteins recognize specific RNA sites? New clues from autogenously regulated ribosomal proteins. Trends Biochem. Sci. 14: 335-338.
- Draper, D.E. (1995) Protein-RNA recognition. Annu. Rev. Biochem. 64: 593-620.
- Draper, D.E. and Reynaldo, L.P. (1999) RNA binding strategies of ribosomal proteins. Nucl. Acids Res. 27: 381-388.
- Dresios, J., Derkatch, I.L., Liebman, S.W., Synetos, D. (2000) Yeast ribosomal protein L24 affects the kinetics of protein synthesis and ribosomal protein L39 improves translational accuracy, while mutants lacking both remain viable. Biochem. 39: 7236-7244.
- Eichler, D.C. and Craig, N. (1994) Processing of eukaryotic ribosomal RNA. Prog. Nucl. Acid Res. Mol. Biol. 49: 197-239.
- Etter, A., Bernard, V., Kenzelmann, M., Tobler, H., Muller, F. (1994) Ribosomal heterogeneity from chromatin diminution in *Ascaris lumbricoides*. Science 265: 954-956.
- Fallon, A.M., Jinks, C.S., Strycharz, G.D., Nomura, M. (1979) Regulation of ribosomal protein synthesis in *Escherichia coli* by selective mRNA inactivation. Proc. Natl. Acad. Sci. 76: 3411-3415.
- Fewell, S.W. and Woolford, J. L. (1999) Ribosomal protein S14 of *Saccharomyces cerevisiae* regulates its expression by binding to RPS14B pre-mRNA and to 18S rRNA. Mol. Cell Biol. 19: 826-834.
- Filippov, V., Solovyev, V., Filippova, M., Gill, S.S. (2000) A novel type of RNase III family proteins in eukaryotes. Gene 245: 213-221.

- Fisher, T., Katcoff, D.J., Achituv, Y. (2004) A phylogenetic study of chthamaloids (Cirripedia; Thoracica; Chthamaloidae) based on 16S rDNA and COI sequence analysis. *Biol. J.* 83: 39-45.
- Frei, C., Galloni, M., Hafen, E., and Edgar, B.A. (2005) The *Drosophila* mitochondrial ribosomal protein mRpL12 is required for Cyclin D/Cdk4-driven growth. *EMBO J.* 24: 623-634.
- Fromont-Racine, M., Senger, B., Saveanu, C., Fasiolo, F. (2003) Ribosome assembly in eukaryotes. *Gene* 313: 17-42.
- Fumagalli, S. and Thomas, G. (2000) S6 phosphorylation and signal transduction. In: N. Sonenberg, J.W.B. Hershey, and M.B. Mathews (Eds.), *Translational Control of Gene Expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 695-718.
- Ganot, P., Bortolin, M.L., Kiss, T. (1997) Site-specific pseudouridine formation in pre-ribosomal RNA is guided by small nucleolar RNAs. *Cell* 89: 799-809.
- Garcia-Marcos, A., Sanchez, S. A., Parada, P., Eid, J., Jameson, D. M., Remacha, M., Gratton, E., Ballesta, J. P. G. (2008) Yeast ribosomal stalk heterogeneity *in vivo* shown by two-photon FCS and molecular brightness analysis. *Biophys. J.* 94: 2884-2890.
- Gerbi, S. and Borovjagin, A.V. (2004) Pre-ribosomal RNA processing in multicellular organisms. In: M. Olson (Ed.), *The Nucleolus*. Landes Bioscience, NY, pp. 170-198.
- Giavalisco, P., Wilson, D., Kreitler, T., Lehrach, H., Klose, J., Gobom, J., Fucini, P. (2005) High heterogeneity within the ribosomal proteins of the *Arabidopsis thaliana* 80S ribosome. *Plant Mol. Biol.* 57: 577-591.
- Gillham, N.W., Boynton, J.E. and Hauser, C.R. (1994) Translational regulation of gene expression in chloroplasts and mitochondria. *Annu. Rev. Genet.* 28: 71-93
- Gonzalez, I.L., Gorski, J.L., Campen, T.J., Dorney, D.J., Erickson, J.M., Sylvester, J.E., Schmickel, R.D. (1985) Variation among human 28S ribosomal RNA genes. *Genetics* 82: 7666-7670.
- Gourse, R.L., Stark, M.J., Dahlberg, A.E. (1982) Site-directed mutagenesis of ribosomal RNA. Construction and characterization of deletion mutants. *J. Mol. Biol.* 159: 397-416.

- Gregory, R.R., Cahill, P.B.F., Thurlow, D.L., Zimmermann, R.A. (1988) Interaction of *Escherichia coli* ribosomal protein S8 with its binding sites in ribosomal RNA and messenger RNA. *J. Mol. Biol.* 204: 295-307.
- Groban, E.S., Narayanan, A., Jacobson, M.P., Conformational changes in protein loops and helices induced by post-translational phosphorylation. *PLoS Comput. Biol.* (2006) 2(e32).
- Guillier, M., Allemand, F., Raibaud, S., Dardel, F., Springer, M., Chiaruttini, C. (2002) Translational feedback regulation of the gene for *L35* in *Escherichia coli* requires binding of ribosomal protein L20 to two sites in its leader mRNA: A possible case of ribosomal RNA–messenger RNA molecular mimicry. *RNA* 8: 878-889.
- Guillier, M., Allemand, F., Dardel, F., Royer, C.A., Springer, M., Chiaruttini, C. (2005) Double molecular mimicry in *Escherichia coli*: binding of ribosomal protein L20 to its two sites in mRNA is similar to its binding to 23S rRNA. *Mol. Microbiol.* 56: 1441-1456.
- Guillier, M., Allemand, F., Raibaud, S., Dardel, F., Springer, M., Chiaruttini, C. (2005b) The N-terminal extension of *Escherichia coli* ribosomal protein L20 is important for ribosome assembly, but dispensable for translational feedback control. *RNA* 11: 728-738.
- Haasen, D., Kohler, C., Neuhaus, G., Merkle, T. (1999) Nuclear export of proteins in plants: AtXPO1 is the export receptor for leucine-rich nuclear export signals in *Arabidopsis thaliana*. *Plant J.* 20: 695-705.
- Halic, M., Becker, T., Pool, M.R., Spahn, C.M., Grassucci, R.A., Frank, J., Beckmann, R. (2004) Structure of the signal recognition particle interacting with the elongation-arrested ribosome. *Nature* 427: 808-814.
- Hancock, J.M. and Dover, G.A. (1988) Molecular coevolution among cryptically simple expansion segments of eukaryotic 26S/28S rRNAs. *Mol. Biol. Evol.* 5: 377-391.
- Hansel, A., Kuschel, L., Hehl, S., Lemke, C., Agricola, H.J., Hoshi, T., Heinemann, S.H. (2002) Mitochondrial targeting of the human peptide methionine sulfoxide reductase (MSRA), an enzyme involved in the repair of oxidized proteins. *FASEB J.* 16: 911-913.

- Hardy, C.F., Balderes, D., Shore, D. (1992) Dissection of a carboxy-terminal region of the yeast regulatory protein RAP1 with effects on both transcriptional activation and silencing. *Mol. Cell Biol.* 12: 1209-1217.
- Hariharan, N. and Perry, R.P. (1990) Functional dissection of a mouse ribosomal protein promoter: significance of the polypyrimidine initiator and an element in the TATA-box region. *Proc. Natl. Acad. Sci. USA* 87: 1526-1530.
- Held, W.A., Ballou, B., Mizushima, S., Nomura, M. (1974) Assembly mapping of 30 S ribosomal proteins from *Escherichia coli*. *J. Biol. Chem.* 249: 3103-3111.
- Henderson, S.L. and Sollner-Webb, B. (1990) Enhancers for RNA polymerase I in mouse ribosomal DNA. *Mol. Cell. Biol.* 10: 4970-4973.
- Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., Mullineaux, P.M. (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* 42: 819-832.
- Hirose, T. and Sugiura, M. (2004) Functional Shine-Dalgarno-like sequences for translational initiation of chloroplast mRNAs. *Plant and Cell Phys.* 45: 114-117.
- Ho, J.H. and Johnson, A.W. (1999) *NMD3* encodes an essential cytoplasmic protein required for stable 60S ribosomal subunits in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 19: 2389-2399.
- Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303: 179-180.
- Hulm, J.L., McIntosh, K.B., Bonham-Smith, P.C. (2005) Variations in transcript abundance among the four members of the *Arabidopsis thaliana* ribosomal protein *S15a* gene family. *Plant Sci.* 169: 267-278.
- Jäkel, S. and Görlich, D. (1998). Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *EMBO J.* 17: 4491-4502.
- Jagannathan, I. and Culver, G. (2003) Assembly of the central domain of the 30S ribosomal subunit: roles for the primary binding ribosomal proteins S15 and S8. *J. Mol. Biol.* 330: 373-383.
- Jenni, S. and Ban, N. (2003) The chemistry of protein synthesis and voyage through the ribosomal tunnel. *Curr. Opin. Struct. Biol.* 13: 212-219.

- Jinks-Robertson, S. and Nomura, M. (1982) Ribosomal protein S4 acts in trans as a translational repressor to regulate expression of the alpha operon in *Escherichia coli*. J. Bacteriol. 1: 193-202.
- Johnson A.W., Lund E., Dahlberg J. (2002) Nuclear export of ribosomal subunits. Trends Biochem. Sci. 27: 580-585.
- Johnson, S. and Warner, J. (1987) Phosphorylation of the *Saccharomyces cerevisiae* equivalent of ribosomal protein S6 has no detectable effect on growth. Mol. Cell. Biol. 7: 1338-1345.
- Kalurachchi, K., Uma, K., Zimmermann, R.A., Nikonowicz, E.P. (1997) Structural features of the binding site for ribosomal protein S8 in *Escherichia coli* 16S rRNA defined using NMR spectroscopy. Proc. Natl. Acad. Sci. USA 94: 2139-2144.
- Kenmochi, N., Kawaguchi, T., Rozen, S., Davis, E., Goodman, N., Hudson, T.J., Tanaka, T., David, C. (1998) A Map of 75 human ribosomal protein genes. Gen. Res. 8: 509-523.
- Kennelly, P. J. (2002) Protein kinases and protein phosphatases in prokaryotes: a genomic perspective. FEMS Microbiol. Lett. 206: 1-8.
- Kenny, B. and Finlay, B.B. (1997) Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C- γ 1. Infect. Immun. 65: 2528-2536.
- Kim, Y-H., Ishikawa, D., Ha, H.P., Sugiyama, M., Kaneko, Y., Harashima, S. (2006) Chromosome XII context is important for rDNA function in yeast. Nucl. Acids Res. 34: 2914-2924.
- Kiss-László, Z., Henry, Y., Bachellerie, J-P., Caizergues-Ferrer, M., Kiss, T. (1996) Site-specific ribose methylation of pre-ribosomal RNA: A novel function for small nucleolar RNAs. Cell 85: 1077-1088.
- Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M. (2001) rrndb: the ribosomal RNA operon copy number database. Nucl. Acids Res. 29: 181-184.
- Klein, D.J., Moore, P.B., Steitz, T.A. (2004) The roles of ribosomal proteins in the structure assembly, and evolution of the large ribosomal subunit. J. Mol. Biol. 340: 141-177.
- Kominami, R., Urano, Y., Mishima, Y., Muramatsu, M. (1981) Organization of ribosomal RNA gene repeats of the mouse. Nucl. Acids Res. 9: 3219-3233.

- Kössel, H., Hoch, B., Maier, R.M., Igloi, G.L., Kudla, J., Zeltz, P., Freyer, R., Neckermann, K., Ruf, S. (1993) RNA editing in chloroplasts. *Plant Mito.* 2: 93-102.
- Krüger, T., Zentgraf, H., Scheer, U. (2007) Intranucleolar sites of ribosome biogenesis defined by the localization of early binding ribosomal proteins. *J. Cell Biol.* 177: 573-578.
- Lafontaine, D.L.J. and Tollervey, D. (2001) Ribosomal RNA. *Nature* 9: 2470-2481.
- Lang, K.J., Kappel, A., Goodall, G.J. (2002) Hypoxia-inducible factor-1alpha mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. *Mol. Biol. Cell* 13: 1792-1801.
- Leader, D.J., Clark, G.P., Watters, J., Beven, A.F., Shaw, P.J., Brown, J.W.S. (1997) Clusters of multiple different small nucleolar RNA genes in plants are expressed as and processed from polycistronic pre-snoRNAs. *EMBO J.* 16: 5742-5751.
- Lee, S-W., Berger, S.J., Martinovic, S., Pasa-Tolic, L., Anderson, G.A., Shen, Y., Zhao, R., Smith, R.D. (2001) Direct mass spectrometric analysis of intact proteins of the yeast large ribosomal subunit using capillary LC/FTICR. *Proc. Natl. Acad. Sci. USA* 99: 5942-5947.
- Lecompte, O., Ripp, R., Thierry, J.C., Moras, D., Poch, O. (2002) Comparative analysis of ribosomal proteins in complete genomes: An example of reductive evolution at the domain scale. *Nucl. Acids Res.* 30: 5382-5390.
- Lescoute, A. and Westhof, E. (2006) The interaction networks of structured RNAs. *Nucl. Acids Res.* 34: 6587-6604.
- Li, Z., Pandit, S., Deutscher, M.P. (1999) Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. *RNA* 5: 139-146.
- Liljas, A. and Al-Karadaghi, S. (1997) Structural aspects of protein synthesis. *Nature Struct. Biol.* 4: 767-771.
- Ling, M.M. and Robinson, B.H. (1997) Approaches to DNA mutagenesis: an overview. *Anal. Biochem.* 254: 157-178.
- Lisitsky, I. and Schuster, G. (1995) Phosphorylation of a chloroplast RNA-binding protein changes its affinity to RNA. *Nucl. Acids Res.* 23: 2506-2511.
- Lodmell, J.S. and Dahlberg, A.E. (1997) A conformational switch in *Escherichia coli* 16S ribosomal RNA during decoding of messenger RNA. *Science* 277: 1262-1267.

- Logan, D.C. and Leaver, C.J. (2000) Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells. *J. Exp. Bot.* 51: 865-871.
- Lu, J.Q., Xu, M.B., Zhou, X.W., Xu, J.G., Tao, Q. (2007) A novel detection of single-stranded DNA binding protein based on ss-DNA modified chip using surface plasmon resonance microscopy. *Chinese Chem. Let.* 18: 441-444.
- Maden, B.E.H. (1990) The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog. Nucl. Acid Res. Mol. Biol.* 39: 241-301.
- Mager, W.H. (1988) Control of ribosomal protein expression. *Biochim. Biophys. Acta* 949: 1-15.
- Marilley, M. and Pasero, P. (1996) Common DNA structural features exhibited by eukaryotic ribosomal gene promoters. *Nucl. Acids Res.* 24: 2204-2211.
- Marquis, D.M., Fahnestock, S.R., Henderson, E., Woo, D., Schwinge, S., Clark, M.W., Lake, J.A. (1981) The L7/L12 stalk, a conserved feature of the prokaryotic ribosome, is attached to the large subunit through its N terminus. *J. Mol. Biol.* 150: 121-132.
- Matsunaga, J., Simons, E.L., Simons, R.W. (1996) RNase III autoregulation: structure and function of rncO, the posttranscriptional “operator”. *RNA* 2: 1228-1240.
- Mattheakis, L. and Nomura, M. (1988) Feedback regulation of the *spc* operon in *Escherichia coli*: Translational coupling and mRNA processing. *J. Bacteriol.* 170: 4482-4492.
- Mauro, V.P. and Edelman, G.M. (2002) The ribosome filter hypothesis. *Proc. Natl. Acad. Sci. USA* 99: 12031-12036.
- McIntosh, K.B. and Bonham-Smith, P.C. (2005) The two ribosomal protein *L23A* genes are differentially transcribed in *Arabidopsis thaliana*. *Genome* 48: 443-454.
- McIntosh, K.B. and Bonham-Smith, P.C. (2006) Ribosomal protein gene regulation: what about plants? *Can. J. Bot.* 84: 342-362.
- McStay, B. (2006) Nucleolar dominance: a model for rRNA gene silencing. *Gen. Dev.* 20: 1207-1214.
- Merianos, H.J., Wang, J., Moore, P.B. (2004) The structure of a ribosomal protein S8/*spc* operon mRNA complex. *RNA* 10: 954-964.
- Merrick, W.C. (2003) Initiation of protein biosynthesis in eukaryotes. *Biochem. Mol. Biol. Ed.* 31: 378-385.

- Michot, B. and Bachellerie J.P. (1991) Secondary structure of the 5' external transcribed spacer of vertebrate pre-rRNA. Presence of phylogenetically conserved features. *Eur. J. Biochem.* 195: 601-609.
- Mitrovich, Q.M. and Anderson, P. (2000) Unproductively spliced ribosomal protein mRNAs are natural targets of mRNA surveillance in *C. elegans*. *Genes Dev.* 14: 2173-2184.
- Mitsui, K., Nakagawa, T., Tsurugi, K. (1989) The Gene and the primary structure of acidic ribosomal protein AO from yeast *Saccharomyces cerevisiae* which shows partial homology to bacterial ribosomal protein L10. *J. Biochem.* 106: 223-227.
- Moehle, C.M. and Hinnebusch, A.G. (1991) Association of RAP1 binding sites with stringent control of ribosomal protein gene transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 11: 2723-2735.
- Morgan, D.G., Menetret, J.F., Radermacher, M., Neuhofer, A., Akey, I.V., Rapoport, T.A., Akey, C.W. (2000) A comparison of the yeast and rabbit 80S ribosome reveals the topology of the nascent chain exit tunnel, inter-subunit bridges and mammalian rRNA expansion segments. *J. Mol. Biol.* 301: 301-321.
- Morimoto, T., Suzuki, Y., Yamaguchi, I. (2002) Effects of partial suppression of ribosomal protein S6 on organ formation in *Arabidopsis thaliana*. *Bio. Biotech. Biochem.* 66: 2437-2443.
- Morita-Yamamuro, C., Tsutsui, T., Tanaka, A., Yamaguchi, J. (2004) Knock-out of the plastid ribosomal protein S21 causes impaired photosynthesis and sugar-response during germination and seedling development in *Arabidopsis thaliana*. *Plant Cell Physiol.* 45: 781-788.
- Muto, A., Ehresmann, C., Fellner, P., Zimmermann, R.A. (1974) RNA-protein interactions in the ribosome, I: Characterization and ribonuclease digestion of 16S RNA-ribosomal protein complexes. *J. Mol. Biol.* 86: 411-432.
- Neigebom, L. and Warner, J.R. (1990) Expression of yeast 5S RNA is independent of the rDNA enhancer region. *Nucl. Acids. Res.* 18: 4179-4184.
- Nevskaya, N., Tishchenko, S., Nikulin, A., al-Karadaghi, S., Liljas, A., Ehresmann, B. (1998). Crystal structure of ribosomal protein S8 from *Thermus thermophilus* reveals a high degree of structural conservation of a specific RNA binding site. *J. Mol. Biol.* 279: 233-244.

- Nilsson, J., Sengupta, J., Frank, J., Nissen, P. (2004) Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. *EMBO Report* 5:1137-1141.
- Nilsson, J., Sengupta, J., Gursky, R., Nissen, P., Frank, J. (2007) Comparison of fungal 80S ribosomes by cryo-EM reveals diversity in structure and conformation of rRNA expansion segments. *J. Mol. Biol.* 369: 429-438.
- Nissen, P., Hansen, J., Ban, N., Moore, P.B., Steitz, T.A. (2000) The structural basis of ribosome activity in peptide bond synthesis. *Science* 289: 920-930.
- Nitta, I., Ueda, T., Watanabe, K. (1998) Possible involvement of *Escherichia coli* 23S ribosomal RNA in peptide bond formation. *RNA* 4: 257-267.
- Noller, H.F. (2005) Reading the ribosome. *Science* 309: 1508-1514.
- Noller, H.F. (2001) Crystal structure of the ribosome at 5.5 Å resolution. *Science* 292: 883-896.
- Noller, H.F. (1984) Structure of ribosomal RNA. *Ann. Rev. Biochem.* 53: 119-162.
- Noller, H.F., Cate, J., Dallas, A., Culver, G., Earnest, T.N., Green, R., Holmberg, L., Joseph, S., Lancaster, L., Lieberman, K., Merryman, C., Newcomb, L., Samaha, R., von Ahlsen, U., Yusupov, M., Yusupova, G., Wilson, K. (2000) Studies on the structure and function of ribosomes by combined use of chemical probing and X-ray crystallography. In: R.A. Garrett, S.R. Douthwaite, A. Liljas, A.T. Matheson, P.B. Moore, H.F. Noller (Eds.), *The Ribosome: Structure, Function, Antibiotics, and Cellular Interactions*. ASM Press, Washington, DC, pp. 129-150.
- Noller, H.F., Hoffarth, V., Zimniak, L. (1992) Unusual resistance of peptidyl transferase to protein extraction procedures. *Science* 256: 1416-1419.
- Nomura, M., Yates, J.L., Dean, D., Post, L.E. (1980) Feedback regulation of ribosomal protein gene expression in *Escherichia coli*: Structural homology of ribosomal RNA and ribosomal protein mRNA. *Proc. Natl. Acad. Sci. USA* 77: 7084-7088.
- Oakes, M.L., Johzuka, K., Vu, L., Eliason, K., Nomura, M. (2006) Expression of rRNA genes and nucleolus formation at ectopic chromosomal sites in the yeast *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 26: 6223-6238.

- Olins, P.O. and Nomura, M. (1981) Translational regulation by ribosomal protein S8 in *Escherichia coli*: structural homology between rRNA binding site and feedback target on mRNA. Nucl. Acids Res. 9: 1757-1764.
- Orr, J.W., Hagerman, P.J., Williamson, J.R. (1998) Protein and Mg^{2+} induced conformational changes in the S15 binding site of 16 S ribosomal RNA. J. Mol. Biol. 275: 453-464.
- Pederson, T. and Politz, J.C. (2000) The nucleolus and the four ribonucleoproteins of translation. J. Cell Biol. 148: 1091-1096.
- Piekna-Przybylska, D., Decatur, W.A., Fournier, M.J. (2007) New bioinformatic tools for analysis of nucleotide modifications in eukaryotic rRNA. RNA 13: 305-312.
- Pirkkala, L. and Sistonen, L. (1999) Antibody supershift assay is inadequate for determining HSF stoichiometry in HSE complexes. Cell Stress Chaper. 4: 259-261.
- Planta, R.J. and Raue, H.A. (1988) Control of ribosome biogenesis in yeast. Trends Genet. 4: 64-68.
- Pontes, O., Lawrence, R.J., Neves, N., Silva, M., Lee, J-H., Chen, Z.J., Viegas, W., Pikaard C.S. (2003) Natural variation in nucleolar dominance reveals the relationship between nucleolus organizer chromatin topology and rRNA gene transcription in Arabidopsis. Proc. Natl. Acad. Sci. USA 100: 11418-11423.
- Powers, T. and Walter, P. (1999) Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in *Saccharomyces cerevisiae*. Mol. Biol. Cell 10: 987-1000.
- Query, C.C., Bentley, R.C., Keene, J.D. (1989) A common RNA recognition motif identified within a defined U1 RNA binding domain of the 70K U1 snRNP protein. Cell 57: 89-101.
- Radimerski, T., Mini, T., Schneider, U., Wettenhall, R., Thomas, G., Jenö, P. (2000) Identification of insulin-induced sites of ribosomal protein S6 phosphorylation in *Drosophila melanogaster*. Biochemistry 39: 5766-5774.
- Ramagopal, S. (1992) Are eukaryotic ribosomes heterogeneous? Affirmations on the horizon. Biochem. Cell Biol. 70: 269-272.
- Rogers, S.O. and Bendich, A.J. (1987) Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol. Biol. 9: 509-520.
- Rosenshine, I., Ruschkowski, S., Stein, M., Reinscheid, D.J., Mills, S.D., Finlay, B.B.

- (1996) A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. *EMBO J.* 15: 2613-2624.
- Rotenberg, M. O. and J. L. Woolford, Jr. (1986) Tripartite upstream promoter element essential for expression of *Saccharomyces cerevisiae* ribosomal protein genes. *Mol. Cell. Biol.* 6: 674-687.
- Rouquette, J., Choismel V., Gleizes, P-E. (2005) Nuclear export and cytoplasmic processing of precursors to the 40S ribosomal subunits in mammalian cells. *EMBO J.* 24: 2862-2872.
- Saez-Vasquez, J., Caparros-Ruiz, D., Barneche, F., Echeverria, M. (2004). A plant snoRNP complex containing snoRNAs, fibrillarin, and nucleolin-like proteins is competent for both rRNA gene binding and pre-rRNA processing *in vitro*. *Mol. Cell. Biol.* 24: 7284-7297.
- Samaha, R.R, Green, R., Noller, H.F. (1995) A base pair between tRNA and 23S rRNA in the peptidyl transferase centre of the ribosome. *Nature* 377: 309-314.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
- Saveanu, C., Fromont-Racine, M., Harington, A., Ricard, F., Namane, A., Jacquier, A. (2001) Identification of 12 new yeast mitochondrial ribosomal proteins including 6 that have no prokaryotic homologues. *J. Biol. Chem.* 276: 15861-15867.
- Savino, T.M., Gébrane-Younès, J., De Mey, J., Sibarita, J-B., Hernandez-Verduna, D. (2001) Nucleolar assembly of the rRNA processing machinery in living cells. *J. Cell Biol.* 153: 1097-1110.
- Schäfer, T., Strauszlig D., Petfalski, E., Tollervey D., Hurt, E. (2003) The path from nucleolar 90S to cytoplasmic 40S pre-ribosomes. *EMBO J.* 22: 1370-1380.
- Scherly, D., Boelens, W., van Venrooij, W.J., Dathan, N.A., Hamm, J., Mattaj, I.W. (1989) Identification of the RNA binding segment of human U1A protein and definition of its binding site on U1 snRNA. *EMBO J.* 8: 4163-4170.
- Schlutzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F. (2000) Structure of functionally activated small ribosomal subunit at 3.3 Å resolution. *Cell* 102: 615-623.

- Schnare, M.N., Damberger, S.H., Gray, M.W., Gutell, R.R. (1996) Comprehensive comparison of structural characteristics in eukaryotic cytoplasmic large subunit (23 S-like) ribosomal RNA. *J. Mol. Biol.* 256: 701-719.
- Schuster, W. and Brennicke, A. (1994) The plant mitochondrial genome: Physical structure, information content, RNA editing, and gene migration to the nucleus. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 45: 61-78.
- Schuwirth, B.S., Borovinskaya, M.A., Hau, C.A., Zhang, W., Vila-Sanjurjo, A., Holton, J.M., Doudna Cate, J.H. (2005) Structures of the bacterial ribosome at 3.5 Å resolution. *Science* 310: 827-834.
- Selmer, M., Dunham, C.M., Murphy, F.V., Weixlbaumer, I.V.A., Petry, S., Kelley, A.C., Weir, J.R., Ramakrishnan, V. (2006) Structure of the 70S ribosome complexed with mRNA and tRNA. *Science* 313: 1935-1942.
- Semrad, K., Green, R., Schroeder, R. (2004) RNA chaperone activity of large ribosomal subunit proteins from *Escherichia coli*. *RNA* 10: 1855-1860.
- Shen, L.X., Cai, Z., Tinoco, I. (1995) RNA structure at high resolution. *Fed. Am. Soc. Exp. Biol. J.* 9: 1023-1033.
- Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Thodoh, N., Shimada, H., Sugiura, M. (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO J.* 5: 2043-2049.
- Sickmann, A. and Meyer, H.E. (2001) Phosphoamino acid analysis. *Proteomics* 1: 200-206.
- Skinner, R.H., Stark, M.J., Dahlberg, A.E. (1985) Mutations within the 23S rRNA coding sequence of *E. coli* which block ribosome assembly. *EMBO J.* 4: 1605-1608.
- Soltis, P.S., Soltis, D.E., Wolf, P.G., Nickrent, D.L., Chaw, S.M., Chapman, R.L. (1999) The phylogeny of land plants inferred from 18S rDNA sequences: pushing the limits of rDNA signal? *Mol. Biol. Evol.* 16: 1774-1784.
- Spahn, C.M.T., Beckmann, R., Eswar, N., Penczek, P.A., Sali, A., Blobel, G., Frank, J. (2001) Structure of the 80S ribosome from *Saccharomyces cerevisiae*-tRNA-ribosome and subunit-subunit interactions. *Cell* 107: 373-386.

- Spahn, C.M.T., Kieft, J.S., Grassucci, R.A., Penczek, P.A., Zhou, K., Doudna, J.A., Frank, J. (2001b) Hepatitis C virus IRES RNA-induced changes in the conformation of the 40S ribosomal subunit. *Science* 291: 1959-1962.
- Spahn, C.M., Jan, E., Mulder, A., Grassucci, R.A., Sarnow, P., Frank, J. (2004) Cryo-EM visualization of a viral internal ribosome entry site bound to human ribosomes: the IRES functions as an RNA-based translation factor. *Cell* 118: 465-475.
- Stewart, M.J. and Thomas, G. (1994) Mitogenesis and protein synthesis: A role for ribosomal protein S6 phosphorylation? *BioEssays* 16: 809-814.
- Stoldt, M., Wöhnert, J., Görlach, M., Brown, L.R. (1998) The NMR structure of *Escherichia coli* ribosomal protein L25 shows homology to general stress proteins and glutamyl-tRNA synthetases. *EMBO J.* 17: 6377-6384.
- Stothard, J.R., Frame, I.A., Miles, M.A. (1997). An evaluation of four staining methods for the detection of DNA in nondenaturing polyacrylamide gels. *Anal. Biochem.* 253: 262-264.
- Sun, W., Li, G., Nicholson, A.W. (2004) Mutational analysis of the nuclease domain of *Escherichia coli* ribonuclease III. Identification of conserved acidic residues that are important for catalytic function *in vitro*. *Nucl. Acids Res.* 33 : 807-815.
- Sweeney, R., Chen, L., Yao, M.C. (1994) An rRNA variable region has an evolutionarily conserved essential role despite sequence divergence. *Mol. Cell. Biol.* 14: 4203-4215.
- Szick-Miranda, K. and Bailey-Serres, J. (2000) Regulated heterogeneity in 12-kDa P-protein phosphorylation and composition of ribosomes in Maize (*Zea mays*) *J. Biol. Chem.* 276: 10921-10928.
- Tasheva, E.S. and Roufa, D.J. (1995) Regulation of human RPS14 transcription by intronic antisense RNAs and ribosomal protein S14. *Genes Dev.* 9: 304-316.
- Tate, W.P. and Poole, E.S. (2004) The ribosome: lifting the veil from a fascinating organelle. *BioEssays* 26: 582-588.
- Taylor, M.A., Mad Arif, S.A., Pearce, S.R., Davies, H.V., Kumar, A., George, L.A. (1992) Differential expression and sequence analysis of ribosomal protein genes induced in stolon tips of potato (*Solanum tuberosum*) during the early stages of tuberization. *Plant Physiol.* 100: 1171-1176.

- Terada, N., Patel, H., Takase, K., Kohno, K., Nairn, A., Gelfand, E. (1994) Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc. Natl Acad. Sci. USA* 91: 11477-11481.
- Theobald, D.L., Mitton-Fry, R.M., Wuttke, D.S. (2003) Nucleic acid recognition by OB-fold proteins. *Annu. Rev. Biophys. Biomol. Struct.* 32: 115-133.
- Thompson, J.M., Musters, W., Cundliffe, E., Dahlberg, A.E. (1993) Replacement of the L11 binding region within *E. coli* 23 S ribosomal RNA with its homolog from yeast: *in vivo* and *in vitro* analysis of hybrid ribosomes altered in the GTPase center. *Eur. J. Biochem.* 12: 1499-1504.
- Timmers, A.C.J., Stuger, R., Schaap, P.J., van't Riet, J., Raue, H.A. (1999) Nuclear and nucleolar localization of *Saccharomyces cerevisiae* ribosomal protein S22 and S25. *FEBS Lett.* 452: 335-340.
- Tishchenko, S.V., Vassilieva, J.M., Platonova, O.B., Serganov, A.A., Fomenkova, N.P., Mudrik, E.S., Piendl, W., Ehresmann, C., Ehresmann, B., Garber, M.B. (2001) Isolation, crystallization, and investigation of ribosomal protein S8 complexed with specific fragments of rRNA of bacterial or archaeal origin. *Biochemistry* 66: 984-953.
- Tremousaygue, D., Manevski, A., Bardet, C., Lescure, N., Lescure, B. (1999) Plant interstitial telomere motifs participate in the control of gene expression in root meristems. *Plant J.* 20: 553-561.
- Tremousaygue, D., Garnier, L., Bardet, C., Dabos, P., Harve, C., Lescure, B. (2003) Internal telomeric repeats and 'TCP domain' protein-binding sites co-operate to regulate gene expression in *Arabidopsis thaliana* cycling cells. *Plant J.* 33: 957-966.
- Tsang, J.S., Henry, Y.A., Chambers, A., Kingsman, A.J., Kingsman, S.M. (1990) Phosphorylation influences the binding of the yeast RAP1 protein to the upstream activating sequence of the PGK gene. *Nucl. Acids Res.* 18: 7331-7337.
- Tsukiyama-Kohara, K., Iizuka, N., Kohara, M., Nomoto, A. (1992) Internal ribosome entry site within hepatitis C virus RNA. *J. Virol.* 66: 1476-1483.
- van Beekvelt, C.A., Kooi, E.A., Graaff-Vincent, M., van't Riet, J., Venema J., Raue, H.A. (2000) Domain III of *Saccharomyces cerevisiae* 25S ribosomal RNA: Its role in binding of ribosomal protein L25 and 60 S subunit formation. *J. Mol. Biol.* 296: 7-17.

- van de Peer, Y., De Rijk, P., Wuyts, J., Winkelmans, T., De Wachter, R. (2000)
The European small subunit ribosomal RNA database. *Nucl. Acids Res.* 28: 175-176.
- Vanderhaeghen, R., De Clercq, R., Karimi, M., Van Montagu, M., Hilson, P., Van
Lijsebettens, M. (2006) Leader sequence of a plant ribosomal protein gene with
complementarity to the 18S rRNA triggers *in vitro* cap-independent translation. *FEBS
Lett.* 580: 2630-2636.
- van Gelder, C.W.G., Gunderson, S.I., Jansen, E.J.R., Boelens, W.C., Polycarpou-Schwartz,
M., Mattaj, I.W., van Venrooij, W.J. (1993) A complex secondary structure in U1A
pre-mRNA that binds two molecules of U1A protein is required for regulation of
polyadenylation. *EMBO J.* 12: 5191-5200.
- Vanrobays, E., Gelugne, J.P., Gleizes, P.E., Caizergues-Ferrer, M. (2003) Late cytoplasmic
maturation of the small ribosomal subunit requires RIO proteins in *Saccharomyces
cerevisiae*. *Mol. Cell Biol.* 23: 2083-2095.
- Varani, C., Cheong, C., Tinoco, Jr. (1991) Structure of an unusually stable RNA hairpin.
Biochemistry 30: 3280-3289.
- Varani, G. and Nagai, K. (1998) RNA recognition by RNP proteins during RNA processing.
Annu. Rev. Biophys. Biomol. Struct. 27: 407-445.
- Venema, J. and Tollervey, D. (1999) Ribosome synthesis in *Saccharomyces cerevisiae*. *Ann.
Rev. Genet.* 33: 261-311.
- Verrier, S.B. and Jean-Jean, O. (2000) Complementarity between the mRNA 5' untranslated
region and 18S ribosomal RNA can inhibit translation. *RNA* 6: 584-597.
- Vignais, M.L., Woudt, L.P., Wassenaar, G.M., Mager, W.H., Sentenac, A., Planta, R.J.
(1987) Specific binding of TUF factor to upstream activation sites of yeast ribosomal
protein genes. *EMBO J.* 6: 1451-1457.
- Vila, A., Viril-Farley, J., Tapprich, W.E. (1994) Pseudoknot in the central domain of small
subunit ribosomal RNA is essential for translation. *Proc. Natl. Acad. Sci. USA* 91:
11148-11152.
- Wakao, H., Romby, P., Ebel, J.P., Grunberg-Manago, M., Ehresmann, C., Ehresmann, B.
(1991) Topography of the *Escherichia coli* ribosomal 30S subunit-initiation factor 2
complex. *Biochimie* 73: 991-1000.

- Wang, C., Sarnow, P., Siddiqui, A. (1993) Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. *J. Virol.* 67: 3338-3344.
- Wang, X., Kikuchi, T., Rikihisa, Y. (2007) Proteomic identification of a novel *Anaplasma phagocytophilum* DNA binding protein that regulates a putative transcription factor. *J. Bacteriol.* 189: 4880-4886.
- Warner, J.R. (2001) Nascent ribosomes. *Cell* 107: 133-136.
- William, M., Clemons, Jr., May, J.L.C., Wimberly, B.T., McCutcheon, J.P., Capel, M.S., Ramakrishnan, V. (1999) Structure of a bacterial 30S ribosomal subunit at 5.5 Å resolution. *Nature* 400: 833-840.
- Williams, A.J., Werner-Fraczek, J., Chang, I.F., Bailey-Serres, J. (2003) Regulated phosphorylation of 40S ribosomal in root tips of maize. *Plant Physiol.* 132: 2086-2097.
- Willis, I.M. (1993) RNA polymerase III. Genes, factors and transcriptional specificity. *Eur. J. Biochem.* 212: 1-11.
- Wilson, K.S. and Noller, H.F. (1998) Mapping the position of translational elongation factor EF-G in the ribosome by directed hydroxyl radical probing. *Cell* 92: 131-139.
- Wilson, J.E., Powell, M.J., Hoover, S.E., Sarnow, P. (2000) Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites. *Mol. Cell. Biol.* 20: 4990-4999.
- Wimberly, B.T., Brodersen, D.E., Clemons, W.M. Jr, Morgan-Warren, R.J., Carter, A.P., Vornrhein, C., Hartsch, T., Ramakrishnan, V. (2000) Structure of the 30S ribosomal subunit. *Nature* 407: 327-339.
- Wittmann-Liebold, B., Köpke, A.K.E., Arndt, E., Krömer, W., Hatakeyama T., Wittmann, H.G. (1990) Sequence comparison and evolution of ribosomal proteins and their genes. In: W.E. Hill (Ed.), *The Ribosome: Structure, Function, and Evolution*, American Society of Microbiology. Washington, DC, pp. 598-616.
- Wool, I.G. (1996) Extraribosomal functions of ribosomal proteins. *Trends Biochem. Sci.* 21: 164-165.
- Wool, I.G., Chan, Y.L., Gluck, A. (1995) Structure and evolution of mammalian ribosomal proteins. *Biochem. Cell Biol.* 73: 933-947.

- Wool, I.G., Chan, Y.L., Gluck, A., (1996) Mammalian ribosome: The structure and the evolution of the proteins. In: J.W.B. Hershey, B.M. Mathews, N. Sonenberg (Eds.), Translational Control. Cold Spring Harbour Laboratory Press, pp. 685-718.
- Woolford, J.L. Jr. (1989) Nuclear pre-mRNA splicing in yeast. *Yeast* 5: 439-457.
- Worton, R.G., Sutherland, J., Sylvester, J.E., Willard, H.F., Bodrug, S., Dube, I., Duff, C., Kean, V., Ray, P.N., Schmickel, R.D. (1998) Human ribosomal RNA genes: orientation of the tandem array and conservation of the 5-prime end. *Science* 239: 64-68.
- Woudt, L.P., Mager, W.H., Nieuwint, R.T., Wassenaar, G.M., van der Kuyl, A.C., Murre, J.J., Hoekman, M.F., Brockhoff, P.G., Planta, R.J. (1987) Analysis of upstream activation sites of yeast ribosomal protein genes. *Nucl. Acids Res.* 15: 6037-6048.
- Wower, I., Kowaleski, M.P., Sears, L.E., Zimmermann, R.A. (1992) Mutagenesis of ribosomal protein S8 from *Escherichia coli*: defects in regulation of the *spc* operon. *J. Bacteriol.* 174: 1213-1221.
- Wower, I.K., Wower, J., Zimmermann, R.A. (1998) Ribosomal protein L27 participates in both 50S subunit assembly and the peptidyl transferase reaction. *J. Biol. Chem.* 273: 19847-19852.
- Wuyts, J., De Rijk, P., Van de Peer, Y., Pison, G., Rousseeuw, P., De Wachter, R. (2000) Comparative analysis of more than 3000 sequences reveals the existence of two pseudoknots in area V4 of eukaryotic small subunit ribosomal RNA. *Nucl. Acids Res.* 28: 4698-4708.
- Xing, Y., Guha Thakurta, D., Draper, D.E. (1997) The RNA binding domain of ribosomal protein L11 is structurally similar to homeodomains. *Nature Struct. Biol.* 4: 24-27.
- Yeh, L-C.C. and Lee, C. (1998) Yeast ribosomal proteins L4, L17, L20, and L25 exhibit different binding characteristics for the yeast 35S precursor rRNA. *Biochim. Biophys. Acta* 1443: 139-148.
- Yoshihama, M., Uechi, T., Asakawa, S., Kawasaki, K., Kato, S., Higa, S., Maeda, N., Minoshima, S., Tanaka, T., Shimizu, N., Kenmochi, N. (2002) The human ribosomal protein genes: sequencing and comparative analysis of 73 genes. *Genome Res.* 12: 379-390.

- Yusupov, M.M., Yusupova, G.Z., Baucom, A., Lieberman, K., Earnest, T.N., Cate, J.H., Noller, H.F. (2001) Crystal structure of the ribosome at 5.5 Å resolution. *Science* 292: 883-896.
- Zanetti, M.E., Chang, I., Gong, F., Galbraith, D.W., Bailey-Serres, J. (2005) Immunopurification of polyribosomal complexes of *Arabidopsis* for global analysis of gene expression. *Plant Physiol.* 138: 624-635.
- Zengel, J.M. and Lindahl, L. (1994) Diverse mechanisms for regulating ribosomal protein synthesis in *Escherichia coli*. *Prog. Nucleic Acid. Res. Mol. Biol.* 47: 331-370.
- Zhao, Y., McIntosh, K.B., Rudra, D., Schawalder, S., Shore, D., Warner, J.R. (2006) Fine-structure analysis of ribosomal protein gene transcription. *Mol. Cell Biol.* 26: 4853-4862.
- Zomerdijk, J.C.B.M. and Tjian, R. (1998) Initiation of transcription on the human rRNA genes. In: M.R. Paule (Ed.), *Transcription of Ribosomal Genes by Eukaryotic RNA Polymerase I*. Landes Bioscience, Austin, TX, pp. 121-134.